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STUDIES ON THE PHYSIOLOGY OF *ASCARIS LUMBRICOIDES*

I. THE RELATION OF THE TOTAL OSMOTIC PRESSURE, CONDUCTIVITY AND CHLORIDE CONTENT OF THE BODY FLUID TO THAT OF THE EXTERNAL ENVIRONMENT

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(*Received 7 March* 1951)

(With Three Text-figures)

Vialli (1923, see Schopfer, 1927), Duval & Courtois (1928) and Schopfer (1924, 1925, 1926, 1927, 1932) have shown by means of freezing-point determinations that the osmotic pressure of the body fluid of *Parascaris megalocephala* and *Ascaris vitulorum* is lower than that of their hosts. Schopfer (1932) found that the depression of the freezing point of extracts of whole specimens of *A. ovis* and *A. lumbricoides* was in each case less than that of the intestinal fluid of the host. He (1932) also found a similar relationship in the case of *Proleptus obtusus*, an intestinal parasite of *Scyliorhinus caniculus*, and Panikkar & Sproston (1941) came to the same conclusion as the result of their work on an unidentified species of *Angusticaecum* from the tortoise. In free-living land or fresh-water nematodes the body fluid is presumably hypertonic to the environment, and Stephenson's work (1942) indicates that in *Rhabditis terrestris* this is, in fact, the case, and that there is an active process of osmotic regulation by means of which this difference in osmotic pressure is maintained.

Schopfer (1925) found that *Parascaris megalocephala* swells or shrinks if the medium with which it is in equilibrium is made more dilute or concentrated. He also found corresponding changes in the freezing-point of the body fluid and thereby showed that water can pass into or out of the body under influence of osmotic forces. Ligaturing experiments demonstrated that this movement of water can take place through the body wall and not merely through the wall of the alimentary canal. Panikkar & Sproston (1941) concluded from their work on *Augusticaecum* that salts, in addition to water, can pass through the body wall. Similar results were obtained by Stephenson (1942) with *Rhabditis terrestris*.

The experiments described in the present paper were designed to throw more light upon the relation between the osmotic pressure of the body fluid of a parasitic nematode, *Ascaris lumbricoides*, and that of the environment.

MATERIAL

The worms were collected from pigs killed at the Harraby Bacon Factory at Carlisle. As soon as possible after the death of the host they were placed in vacuum flasks and sent by passenger train to Newcastle. Immediately they were received they were

washed in warm water and placed in the saline medium, to be described presently, in a thermostat at 37° C. It was unavoidable that there should be some variation in the length of time between the removal of the worms from the host and their arrival at the laboratory, but it was in most cases 5 hr. or less. There was also inevitably some cooling, and the temperature in the flasks when they were received was usually 20–25° C. This temporary lowering of temperature did not seem to cause any serious harm to the worms, which quickly regained their full vigour on being transferred to the warm saline medium. Later experiments showed that it is possible to keep the worms alive and healthy in the laboratory for more than 3 weeks, and, in the case of one individual, for as long as 40 days. It must be emphasized that, in the work to be described, only animals which appeared healthy and normal were used.

METHODS

(*a*) *Measurement of weight*

For the weight measurements the worms were dried rapidly on filter-paper and weighed in air. Batches of about five worms were generally used to reduce the effect of individual variations. These individual variations were appreciable and were partly due to a source of error which could not be controlled, namely, the amount of fluid in the alimentary canal. Some worms on handling eject a considerable volume of fluid from the anus.

(*b*) *Collection of body fluid*

Female worms were rinsed in tap water and dried on filter-paper. The posterior end of each animal was held tightly to prevent ejection of the gut contents, and an incision was made through the body wall about 1 cm. in front of the anus, care being taken not to puncture the alimentary canal. Expulsion of the body fluid was then aided by gently squeezing between the fingers, beginning in the region of the vulva. In certain parts of the work determinations were made on the fluid from a single individual, but, in most experiments, that obtained from a number of worms was used. This was always the case where material was required for freezing-point determinations.

(*c*) *Osmotic pressure*

As a measure of this the depression of the freezing-point was determined. A Beckmann thermometer of the usual size was used which required samples of about 20 ml. of fluid. This quantity of the body fluid could usually be obtained from about twenty-five female worms.

Since there has been much discussion and difference of opinion as to the best method to employ in the determination of the freezing-point of biological fluids it is necessary to describe in detail the procedure which has been used and found most satisfactory. This description will be found in the appendix to the present paper, the results are given in terms of the equivalent concentrations of sodium chloride and are accurate to about 1 mmol.

(*d*) *Conductivity*

In determining the electrical conductivity a Kohlrausch bridge method was used. The apparatus included a valve oscillator, and the circuit for this was very kindly supplied to us by Prof. R. J. Pumphrey.

The determinations were made on diluted body fluid. A sample of about 0·3 ml. was taken with a micro-pipette, diluted to 25 ml. with glass-distilled water, and the resistance measured at 37° C. The apparatus was calibrated by means of standard solutions of sodium chloride which were sampled, diluted and treated in exactly the same way as the body fluid. The reciprocals of the resistances of these solutions were plotted against the concentration and gave a straight-line graph. From this the conductivities of the body fluid samples could be read off in terms of the equivalent concentrations of sodium chloride. The method is accurate to about 1 mmol. concentration of sodium chloride. The values obtained can, however, only be taken as an approximate measure of the total concentration of ions in the body fluid. Complex mixtures such as we are dealing with here do not necessarily behave in the same way as a simple solution of a single salt. Moreover, the dilution of the body fluid may also cause changes in the balance of ions.

(*e*) *Chloride concentrations*

This was determined by Patterson's (1928) modification of the Vohlhard method as further modified by Beadle & Cragg (1940). The micro-pipette, of about 0·3 ml. capacity, was the same as that which was used for the conductivity determinations. Duplicate determinations were made, and the results of a pair were not accepted if they differed by more than 1·5 mmol. The final results are accurate to the nearest 1 mmol. Dr Eden of the Ministry of Agriculture and Fisheries Veterinary Laboratory, Weybridge, very kindly checked certain of our determinations, using a modification of the more elaborate method of Whitehorn (1921). His results agree with ours very closely.

(*f*) *Hydrogen-ion concentration*

This was measured electrometrically by means of a glass electrode and a Cambridge pH meter of the laboratory type. The glass electrode was of the type with a thin flat membrane described by MacInnes & Dole (1929, p. 57). As only a small volume of fluid was available in many cases, the apparatus used was similar to that described and figured by Holden (1938, p. 202), which requires only about 0·2 ml. of fluid.

(*g*) *Special preparations of the worms*

Three types of preparations were used for certain purposes:

(i) Silk ligatures were tied round the worm at a distance of about 1 cm. from each end. The animals withstood this treatment remarkably well and remained alive and apparently healthy for some days.

(ii) Eviscerated cylinders of the body wall were prepared by cutting out a length of about 7 cm. of the posterior two-thirds of the body and removing the reproductive

organs and alimentary canal. One end of the cylinder was then ligatured, the cylinder filled with body fluid from the same animal and the other end ligatured.

(iii) Preparations of the cuticle alone were made by turning an eviscerated cylinder of body wall inside out on a glass rod of suitable diameter. The muscle and the hypodermis were then removed by careful scraping with the finger nail. The cuticle was then washed and turned right side out again.

THE BODY FLUID OF *ASCARIS LUMBRICOIDES* OBTAINED FRESH FROM THE PIG

The body fluid is clear and reddish in healthy worms. About 2 ml. can be obtained from a large individual. In some cases a fine deposit separates on standing. Such samples were not used in the experiments.

The hydrogen-ion concentration seems to be somewhat variable. In one case body fluid obtained from a batch of eight worms from the same host was sampled about 4 hr. after removing from the host. During this period the animals had been exposed to intestinal fluid from the host and kept at a temperature of 37° C. Special care was taken to determine the pH immediately the body fluid was obtained. The eight worms gave individual values varying from pH 6·7 to 6·9 with an average of 6·79. Another batch of five worms from another host received on the same day and treated in the same way varied from pH 6·5 to 7·0, with an average value of 6·78.

This considerable variation in the hydrogen-ion concentration of the body fluid is partly, at any rate, due to exposure to air, although, in the determinations quoted above, this was reduced as far as possible. In the case of one large individual the body fluid was left exposed to the air in a watch-glass and samples removed at intervals of 3 min. The pH values showed a continuous rise from 6·9 initially to 7·13 at 15 min. after 'bleeding'. This was confirmed by further observations. The decrease in hydrogen-ion concentration is presumably due to the loss of volatile acid from the body fluid.

The results of determinations of the total osmotic concentration of the body fluid as calculated from the depression of the freezing-point are given in Table 1. The

Table 1. *Body fluid of* Ascaris lumbricoides

Date	No. of worms in sample	Δ ° C.	Total osmotic concentration expressed in terms of equivalent concentrations of NaCl (mmol.)	Total ionic concentration calculated from conductivity expressed in terms of equivalent concentrations of NaCl (mmol.)	Chloride concentration (mmol.)
26. v. 42	*c.* 50	0·625	182	139	48
2. vi. 42	*c.* 50	0·682	200	146	56
7. vii. 42(*a*)	46	0·627	183	140	53
7. vii. 42(*b*)	27	0·652	191	146	54
14. vii. 42(*a*)	39	0·662	194	142	54
14. vii. 42(*b*)	23	0·681	199	143	51
18. vii. 42	12	—	—	140	51
Mean (unweighted)		0·655	192	142	52

data derived from conductivity measurements expressed in terms of the equivalent concentrations of sodium chloride, and the figures for the chloride concentration of the body are also included in the same table. The determinations given in Table 1 were made on mixed samples of the body fluid obtained from a number of worms.

The results of the determinations made on the intestinal contents of the pig are given in Table 2. In some cases these were made upon the fluid obtained from a single animal, and in others approximately equal volumes from several pigs were mixed.

Table 2. *The intestinal fluid of the pig*

Date	No. of pigs from which sample was obtained	Δ ° C.	Total osmotic concentration expressed in terms of equivalent concentrations of NaCl (mmol.)	Total ionic concentration calculated from conductivity expressed in terms of equivalent concentrations of NaCl (mmol.)	Chloride concentration (mmol.)
14. viii. 42	2	0·773	227	167	102
18. viii. 42	1	0·905	265	164	69
22. viii. 42	1	0·806	238	159	85
25. viii. 42 (*a*)	1	0·920	270	176	44
25. viii. 42 (*b*)	1	0·940	276	185	79
5. i. 43 (*a*)	5	—	—	184	39
5. i. 43 (*b*)	5	—	—	169	34
5. i. 43 (*c*)	5	—	—	166	50
5. i. 43 (*d*)	5	—	—	180	85
5. i. 43 (*e*)	5	—	—	186	54
5. i. 43 (*f*)	5	—	—	180	86
Mean (unweighted)	—	0·869	257	174	66

Schopfer (1925) has shown that the osmotic pressure of the intestinal fluid of the horse increases rapidly when the samples stand at room temperature. We have found that this is also true for the fluid from the pig, and that the conductivity likewise increases. All the determinations of osmotic pressure quoted in Table 2 were therefore made within an hour of the death of the pig or upon samples which had been kept for an additional 18 hr. frozen solid. Previous experiments showed that freezing for this length of time produced no appreciable change in the results (although see p. 20). All samples were analysed as soon as possible after being taken from the pig.

It should be noted that the samples used for these determinations may not be quite typical. Some samples could not be used, since they turned into a jelly-like mass at room temperature and could not be centrifuged.

As might be expected, there is much variation between the samples. This is particularly marked in the case of the chloride concentrations which, on the average, are closer to Schopfer's (1924) figures of 80 mmol. for horse intestinal fluid than to those of Duval & Courtois (1928), who obtained a mean value of 43 mmol. for material from the same animal. In the case of the pig, at any rate, the variation between individual samples is so great that little significance can be attached to the mean value of the chloride concentration.

The values for the electrical conductivity ('total ionic concentration' in Table 2) and for osmotic pressure are much less variable. The latter agree fairly well with Schopfer's (1927) figures for the same material (Δ0·9–1·0° C.).

The variability of these figures is in accordance with expectation and many factors are involved, such as the nature of the food of the pig and the time of the last meal, the amount of drinking water available and the differences between one animal and another. The results serve to emphasize that an intestinal parasite such as *A. lumbricoides* does not live in a constant but in a variable environment.

THE EFFECTS OF IMMERSION IN A SALINE MEDIUM

In order to keep the worms for any considerable length of time in the laboratory they must be immersed in a fluid medium and kept at a temperature close to that of their normal environment. The ideal medium would be, presumably, the intestinal fluid of the pig, but, owing to bacterial action, it is impossible to use this for more than a very short period. Purely saline media have the disadvantage that the whole of the osmotic pressure is due to inorganic salts. Many of the constituents of the worm's normal environment are absent, and the concentration of some of the inorganic ions has to be raised above the level to which the worm is accustomed.

At the time the work described in the present paper was being carried out, little information was available regarding the electrolyte composition of either the body fluid of *Ascaris* or the intestinal fluid of the pig. In spite of the objections mentioned above it was decided to use a saline medium, and sea water, suitably diluted, was chosen as providing a convenient, balanced salt solution. This medium differs somewhat from mammalian blood in the relative concentrations of its cations, but there is no reason to suppose that there is any close similarity in the electrolyte composition of the latter fluid and the intestinal contents. In fact, as McCance (1936) points out, 'although the gastro-intestinal fluids... have the same osmotic pressure as the serum, they are true secretions in that their ionic pattern is different'.

One marked difference between the inorganic electrolyte composition of mammalian blood and that of sea water is in the magnesium content. In human blood serum the concentration of magnesium is about 1 mmol., while in 30% sea water it is about 16 mmol. In a subsequent paper it will be shown that the magnesium of the intestinal contents of the pig, although variable, approaches that of the medium employed in these experiments.

Schopfer (1925) has shown that in *Ascaris* the body shrinks or swells in any medium with which it is not in osmotic equilibrium. Since the osmotic pressure of sea water is considerably higher than that of the normal environment of the worms, the most suitable concentration was determined by placing batches of worms in various dilutions of sea water and weighing the animals at intervals. It is not necessary to give the details of these experiments, which supported Schopfer's observations referred to above and showed that the body weight remained most nearly constant in 29% sea water (i.e. 29 vol. sea water diluted with 71 vol. tap water). In concentrations less than this the body weight increased while shrinkage occurred in stronger

solutions. In practice, 30% sea water was used as the standard saline medium in most of the experiments described in this paper, and in this the worms survive for a considerable time.

THE EFFECT OF LIGATURING ON THE RATE OF SWELLING IN HYPOTONIC SEA WATER

In *Ascaris* there are two main surfaces through which water might pass into or out of the body cavity, the body wall and the alimentary canal. An attempt was made to determine their relative importance.

Table 3. *Swelling of female* Ascaris *exposed to hypotonic sea water* (20%)

Time (hr.)	Ligatured worms				Unligatured worms			
	No. of worms	Total weight (g.)	Average weight (g.)	Increase in weight (%)	No. of worms	Total weight (g.)	Average weight (g.)	Increase in weight (%)
0	45	216·51	4·81	—	45	217·44	4·83	—
14	45	256·94	5·71	18·7	45	276·66	6·15	27·3
20	45	264·49	5·88	22·3	45	280·74	6·39	32·3
37	35	281·26	6·25	30·0	45	296·29	6·59	36·4
44	42	265·82	6·33	31·6	45	299·73	6·66	37·9
62	32	212·25	6·63	37·8	33	225·68	6·84	41·6
68	24	161·10	6·71	39·5	33	227·56	6·90	42·8
86	16	111·96	7·00	45·5	18	130·08	7·27	50·3

In each of these experiments a batch of female worms was divided into two groups, equal in number and, as far as possible, corresponding in the size of the individual animals. The members of one group were ligatured at both ends, while those of the other group were not. In each of a number of covered jars containing 1 l. of 20% sea water at 37° C. were placed five ligatured and five unligatured worms. The medium was renewed each day and the worms were weighed at intervals. The total weight, mean weight and percentage increases in the mean weight of the ligatured and unligatured worms in a typical experiment are given in Table 3 and represented graphically in Fig. 1. The smaller number of worms recorded in the later stages of the experiment is accounted for by the fact that some individuals died or became unhealthy as the result of prolonged exposure to the hypotonic medium.

The experiment shows clearly that ligaturing at both ends decreases the rate at which water is taken up from a hypotonic medium. It is evident that the alimentary canal of the worm is an important surface for water exchange. The possibility that water may enter through the vulva and so by way of the reproductive system is not excluded by these experiments. Similar treatment of males in which the reproductive aperture is necessarily closed by the ligature at the posterior end of the body yields, however, similar results. Both ligatured and unligatured worms swell in 20% sea water, but the rate of increase is greater in the latter than in the former. These experiments demonstrate, therefore, that water can pass into the worm through both the body wall and the alimentary canal.

THE EFFECTS PRODUCED ON THE BODY FLUID BY IMMERSION OF THE WORMS IN VARIOUS CONCENTRATIONS OF SEA WATER

Batches of worms were placed in 20, 25, 30, 35 and 40% sea water for periods ranging from 40 to 87 hr. At the end of the experiment each batch of worms was washed in tap water and dried and the body fluid extracted. Determinations of the osmotic pressure, total ionic concentration and chloride concentration were then made upon the pooled fluid obtained from each batch of worms. The results are summarized in Table 4, from which it can be seen that there is no significant difference between the values obtained after about 40 hr. and after 87 hr. exposure to the

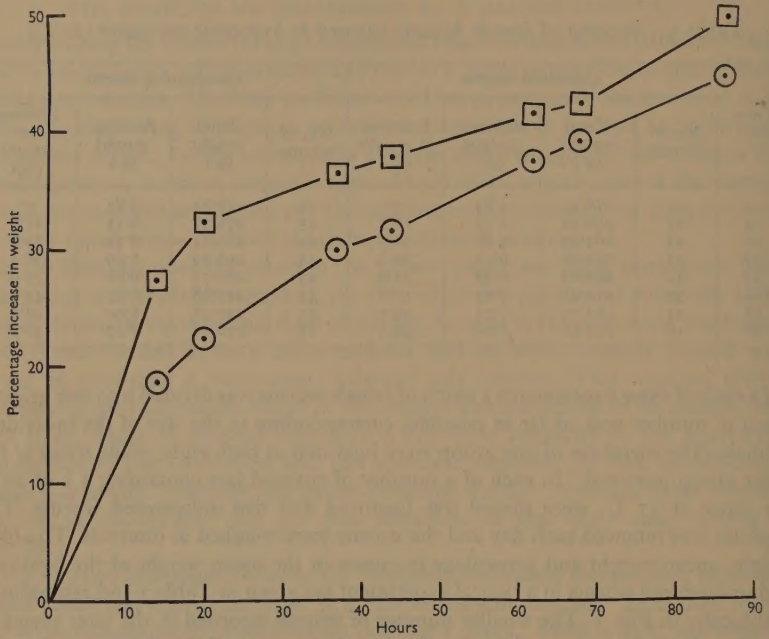

Fig. 1. Increase in weight of normal (⊡) and of ligatured (⊙) female *Ascaris lumbricoides* in 20 % sea-water.

various saline media. It may be concluded, therefore, that after 40 hr. a condition approximating to equilibrium has been reached. It has therefore been considered legitimate to average the results for all times of exposure and the unweighted means are given in the table.

There is a striking contrast to the conditions found when the body fluid of the worm is compared with the normal external medium. The body fluid of *Ascaris* is distinctly hypotonic to the intestinal fluid of the pig. When the worm is transferred to a saline medium in which the body weight remains approximately constant, namely, 30% sea water, the internal osmotic pressure remains almost unchanged but the osmotic gradient is reversed. 30% sea water has an osmotic pressure

corresponding to 176 mmol. NaCl, whereas that of pig intestinal fluid, although variable, is considerably higher, being equivalent to that of about 260 mmol. NaCl. As can be seen from Table 4 and Fig. 2 the variation of the osmotic pressure of the body fluid of the worm with that of the external saline medium nearly corresponds with that which would be expected if we were dealing with an animal enclosed

Table 4. *Body fluid of* Ascaris lumbricoides *after animal exposed to saline medium*

Date	Concentration of sea water (%)	No. of worms in experiment	Length of time of immersion (hr.)	Δ °C.	Total osmotic concentration as equivalent concentration of NaCl (mmol.)	Total ionic concentration as equivalent concentration of NaCl (mmol.)	Chloride concentration (mmol.)
20. viii. 42	20	16	43	0·465	135	97	54
21. viii. 42	20	27	62	0·465	135	93	52
22. viii. 42	20	28	87	0·477	138	97	53
			Mean	0·469	136	96	53
11. ix. 42	25	20	40	0·570	168	117	62
12. ix. 42	25	20	64	0·560	163	115	66
13. ix. 42	25	23	87	0·558	162	112	67
			Mean	0·563	164	115	65
30. vii. 42	30	*c.* 30	46	0·698	204	140	73
6. viii. 42	30	33	44	0·667	195	131	72
15. viii. 42	30	38	87	0·596	174	128	79
29. viii. 42	30	35	87	0·640	187	133	84
11. ix. 42	30	40	40	0·649	189	135	73
12. ix. 42	30	15	64	0·682	200	141	85
13. ix. 42	30	19	87	0·680	199	132	79
			Mean	0·659	193	134	78
3. ix. 42	35	40	40	0·739	216	157	93
4. ix. 42	35	31	63	0·743	217	156	93
5. ix. 42	35	27	87	0·754	221	153	92
			Mean	0·745	218	155	93
10. ix. 42	40	40	43	0·842	247	180	108
11. ix. 42	40	40	63	0·847	248	183	109
12. ix. 42	40	34	87	0·837	244	176	108
			Mean	0·842	246	180	108
External medium: 20% sea water				0·408	118	123	111
30% sea water				0·604	176	185	166
40% sea water				0·802	235	247	220
Body fluid of fresh worms not exposed to saline medium (mean values from Table 1):				0·655	192	142	52
Intestinal fluid of the pig (mean values from Table 1):				0·869	257	174	66

within a completely semi-permeable membrane. This correspondence is not exact. The osmotic pressure of the body fluid of animals immersed in 20% sea water is greater and of those in 40% sea water is less than would be expected on such a hypothesis, but the differences are not large enough to be significant on the figures available. There is, however, much other evidence to show that such a completely semi-permeable membrane is not present.

The data obtained from the measurements of conductivity indicate that no great change takes place in the total ionic concentration of the body fluid when the worm

is removed from its normal environment to 30% sea water; there is a slight fall equivalent to about 8 mmol. NaCl. This occurs in spite of the fact that the conductivity of 30% sea water is slightly greater than that of the intestinal fluid of the pig.

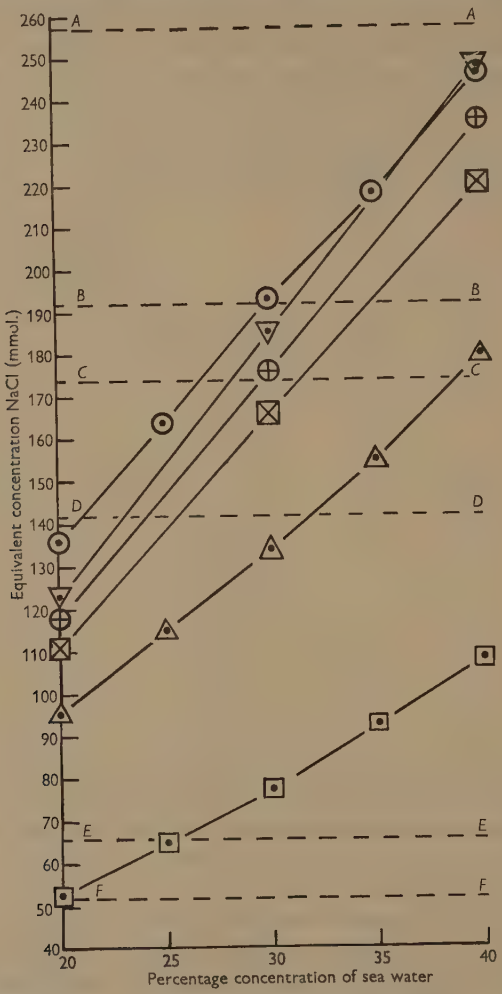

Fig. 2. Total osmotic concentration, total ionic concentration and chloride content of the body fluid of female *Ascaris lumbricoides* after exposure to various concentrations of sea water and corresponding values for the media. All values expressed as equivalent concentrations of sodium chloride. ⊙ osmotic pressure, △ total ionic concentration, ⊡ chloride concentration of body fluid. ⊕ osmotic pressure, ▽ total ionic concentration, ⊠ chloride concentration of medium. The horizontal broken lines mark the levels of the following values: *AA* osmotic pressure, *CC*, total ionic pressure, *EE* chloride concentration of intestinal fluid of pig, *BB* osmotic pressure, *DD* total ionic concentration, *FF* chloride concentration of fresh *Ascaris*.

The conductivity of the body fluid is considerably lower than that of the external medium, whether this is intestinal fluid or one of the solutions used. Comparison of the results obtained from worms immersed in the various strengths of sea water employed shows that there is a fairly close correspondence between the conductivity of the body fluid of the worm and that of its environment, although the decrease in the former is proportionately less at the lower concentrations.

The chloride concentration of the intestinal fluid of the pig is extremely variable, ranging, in the samples we have examined (Table 2), from 34 to 102 mmol., with an average of 66 mmol. That of the body fluid of the fresh worm is fairly constant at about 52 mmol. It is, therefore, doubtful whether there is any significant difference between the internal and external chloride concentrations so long as the worm is exposed to its normal environment. Immersion of the worm in 30% sea water, whose chloride concentration is 166 mmol., increases that of the body fluid to about 78 mmol. In all the concentrations of sea water employed the concentration of chloride in the body fluid is maintained at a value slightly less than half that of the external medium. It will be shown later in this paper that the maintenance of this difference when the worm is exposed to widely different external chloride concentrations is due to an active process of regulation. As will be seen from Table 5 the ratio of the two concentrations is remarkably constant so long as the animals are exposed to a purely saline solution.

Table 5

External medium	Ratio *Ascaris* body fluid/external medium		
	Osmotic pressure	Total ionic concentration	Chloride concentration
20% sea water	1·152	0·781	0·478
25% sea water	1·116	0·747	0·468
30% sea water	1·023	0·724	0·470
35% sea water	1·063	0·718	0·482
40% sea water	1·047	0·729	0·491
Pig intestinal fluid	0·747	0·816	0·788

EXPERIMENTS ON LIGATURED WORMS

Two series of experiments were carried out on female worms ligatured at both anterior and posterior ends.

In the first series the worms had been kept overnight in 29% sea water and were then immersed in 20% sea water. The conductivity and the chloride concentration only were determined, and these measurements were made upon body fluid from individual worms. The results are given in Table 6, and the value given in each case is the mean of each group of worms.

In the second series the worms were immersed in 30% sea water without previous exposure to any medium other than that provided by their normal environment. Larger numbers of worms were used and the body fluid was pooled. Determinations of osmotic pressure, in addition to conductivity and chloride concentration, were made. The results are given in Table 7 and should be compared with those for normal worms in Table 4.

Table 6. *Body fluid of animals exposed to* 20% *sea water*

Date	Length of time of immersion in 20% sea water (hr.)	Ligatured worms				Unligatured worms			
		No. of worms	Total ionic concentration as equivalent concentration of NaCl (mmol.)	No. of worms	Chloride concentration (mmol.)	No. of worms	Total ionic concentration as equivalent concentration of NaCl (mmol.)	No. of worms	Chloride concentration (mmol.)
1. iv. 41	23	6	118	5	57	6	112	6	59
1. iv. 41	28	5	126	5	44	5	117	4	39
2. iv. 41	48	5	104	4	50	6	94	5	49
3. iv. 41	71	6	106	4	34	6	98	6	50
Mean (unweighted)			114	—	46	—	103	—	49

Table 7. *Body fluid of ligatured animals exposed to* 30% *sea water*

Date	No. of worms ligatured	Length of time of immersion in 30% sea-water (hr.)	Δ °C.	Total osmotic concentration as equivalent concentration of NaCl (mmol.)	Total ionic concentration as equivalent concentration of NaCl (mmol.)	Chloride concentration (mmol.)
26. xi. 42	18	43	0·710	206	146	70
28. xi. 42	21	88	0·695	202	137	66
Mean (unweighted)		—		204	142	68

The results of both these series of experiments demonstrate that ligatured worms behave in essentially the same way as those which are not. It is noteworthy, however, that the chloride concentrations in the body fluid are smaller than in normal worms under the same conditions. In any case, it is clear that similar results are obtained when the alimentary canal of the worm is no longer available as a surface for exchange between the animal and its environment.

EXPERIMENTS ON PREPARATIONS OF THE COMPLETE BODY WALL AND OF THE CUTICLE ALONE

The animals from which these preparations were made had been kept previously in 29% sea water. Cylinders were prepared as described on pp. 3–4 and filled with body fluid freshly obtained. They were then placed in 20% sea water for 48 hr., after which the conductivity and the chloride concentration of the fluid contained in the individual preparations were determined. The results are given in Table 8.

The osmotic pressure and the total ionic concentration of 29% sea water are equivalent to 160 and 179 mmol. NaCl respectively. It will be seen that the values obtained for the cuticle preparations, although smaller, are of the same order of magnitude as these, suggesting that the cuticle alone presents little resistance to the passage of electrolytes. With the whole body wall, on the other hand, the total ionic concentration and, especially, the chloride are maintained at much lower values.

THE EFFECT OF LOWERING THE CHLORIDE CONCENTRATION OF THE EXTERNAL MEDIUM

From the evidence which has already been presented it appears that *Ascaris*, when bathed in the intestinal fluid of the pig, maintains the chloride concentration of its body fluid at a value nearly the same as or perhaps slightly below that of the environment. If the worms are placed in a saline medium whose chloride concentration is much higher than that of pig intestinal fluid the rise in internal chloride is

Table 8

Date	Type of preparation	Total ionic concentration as equivalent concentration NaCl (mmol.)	Chloride concentration (mmol.)
10. iv. 42	Complete body wall	133	77
	Complete body wall	117	58
	Complete body wall	135	63
	Complete body wall	—	53
	Complete body wall	—	72
	Cuticle only	164	142
	Cuticle only	158	119
	Cuticle only	160	132
	Cuticle only	160	—

proportionately much smaller. The ratio of internal to external chloride concentration, which is about 0·79 with pig intestinal fluid, falls to about 0·48 with the sea-water media. These facts suggest that *Ascaris* possesses a mechanism for the regulation of the chloride concentration of its body fluid.

It is, therefore, important to discover what happens when the animals are immersed in a saline medium in which the chloride concentration has been reduced while the osmotic pressure remains constant. The medium used was composed of equal volumes of 30% sea water and isotonic sodium nitrate solution. Sodium nitrate was selected because of the similarity of the mobilities of the chloride and nitrate ions. The chloride concentration of this medium was 83 mmol.

The general plan of the various experiments carried out can best be illustrated by quoting a typical one:

Three batches of female worms, each consisting of ten individuals, were placed in 30% sea water for 2 days. At the end of this time the body fluid of all the worms was removed, that obtained from each batch being mixed but kept separate from the other two. Part of each of these three samples was used for the determination of the chloride concentration with the following results:

Batch 1: 68 mmol. batch 2: 73 mmol. batch 3: 75 mmol.

The remainder of the body fluid was used to fill a number of eviscerated cylinders of the body wall (see pp. 3–4). These were ligatured securely at each end and placed in the sea water-$NaNO_3$ medium described above. After 2 days in this medium

measurements of the chloride concentration of the body fluid were made again, and gave the following values for the individual cylinders:

Batch 1: 38, 43, 34 mmol. batch 2: 33, 51 mmol. batch 3: 57, 37, 44 mmol.

A fourth batch of ten individuals was placed in 30% sea water at the same time as the other three. After 2 days they were transferred to the sea water-$NaNO_3$ medium without being operated on in any way. After a further 2 days they were divided into two groups of five worms each and the body fluid removed. In each of these two groups the chloride concentration of the mixed body fluid was 56 mmol.

Similar results were obtained in other experiments, some essentially the same as the one described and some in which the worms were ligatured at the anterior and posterior ends after exposure to 30% sea water but before transference to the sea water-$NaNO_3$ medium.

These experiments show that the chloride concentration of the body fluid, after it has been raised above the normal value by exposing the worms to 30% sea water, is markedly reduced by their transference to a medium of equal osmotic pressure but lower chloride content. In both media the internal chloride concentration which is attained is less than the external. Although the chloride content of the sea water-$NaNO_3$ medium (83 mmol.) is slightly higher than the average value found in the body fluid after 2 days' exposure to 30% sea water, the worms seem to be able to excrete chloride against a concentration gradient. Moreover, this power apparently resides in the body wall, since the openings of the alimentary canal and the excretory system are excluded in the experiments with ligatured worms, and all external apertures in those in which cylinder preparations were used. In all the experiments which have been performed the cylinder preparations have reduced the internal chloride concentration to a greater extent than either ligatured worms or normal animals.

DISCUSSION

The data given in this paper agree with those of the workers already quoted who have shown that the osmotic pressure of the body fluid of a nematode parasitic in the intestine of its host is less than that of the medium to which it is normally exposed. They also support the conclusion of Schopfer (1925) and of Panikkar & Sproston (1941) that the body wall of such a parasite is permeable to water. While the experiments on the increase of weight of female worms ligatured at both ends and exposed to hypotonic solutions do not exclude the reproductive system as a possible surface for the passage of water, those on similarly treated males show that, in this sex at least, the body wall is not impermeable. Comparison with the behaviour of unligatured animals indicates, however, that the alimentary canal is also an important surface for water exchange.

A noteworthy fact, the explanation of which must await further investigation, is that, although the medium in which the change in weight of *A. lumbricoides* is least is approximately 30% sea water, the osmotic pressure of this solution is much lower than that of the intestinal fluid of the pig. The graph (Fig. 2) representing the changes in the osmotic pressure of the body fluid of worms immersed in 20–40% sea water

seems, at first sight, to point to a simple osmotic relation between the body fluid and the external medium. That this is not correct is suggested by two facts. First, as has been mentioned already, the osmotic pressure of the body fluid of the worm is, in all cases investigated, much lower than that of the intestinal contents of the pig and, secondly, the range of variation in the former is less than in the latter. It is impossible to account for a difference in osmotic pressure between the body fluid of the worm and its normal environment equivalent to about 65 mmol. NaCl except by assuming complete impermeability of the body surface or the existence of some active osmoregulatory mechanism. The evidence obtained by previous investigators and that given in the present paper is sufficient to eliminate the first of these two possibilities.

Like that for the osmotic pressure, the graph of the total ionic concentration (as measured by the conductivity) of the body fluid of worms exposed to dilutions of sea water varying from 20 to 40% is almost a straight line. It is not, however, so nearly parallel to that of the external medium (Fig. 2). The ratio of the internal to the external ionic concentration (Table 5) is fairly constant over the range 30–40% sea water and distinctly less than that for fresh worms and intestinal fluid. In 25% and still more in 20% sea water the ratio increases and approaches that which seems to be characteristic of the animals in their normal environment.

The relations between the concentration of chloride in the body fluid and in the external medium are interesting. In the intestinal fluid of the pig the chloride concentration is very variable, e.g. in the samples examined, from 34 to 102 mmol. with an average value of 66 mmol. In the body fluid it is fairly constant, varying from 48 to 56 mmol., with an average value of 52 mmol. Removal of the animals to 30% sea water, which has a chloride concentration of 166 mmol., causes that of the body fluid to rise to about 78 mmol. This is accompanied by a fall in the ratio of the internal to external chloride concentration from about 0·79 to 0·47 (Table 5). It should further be noted that this ratio remains approximately constant over the whole range of sea-water concentrations employed (20–40%).

These data indicate that there is a mechanism for the regulation of the internal chloride concentration. This would not be surprising in view of the great variation in the chloride concentration to which the worms are exposed in their normal environment. Experiments with normal and ligatured worms and with cylinder preparations of the body wall exposed to a medium which is isotonic with 30% sea water but in which half of the chloride has been replaced by nitrate suggest that the animal is able to excrete chloride against a concentration gradient. Moreover, since this effect seems to be more marked in cylinder preparations than in ligatured worms and in ligatured than in normal worms it seems probable that the mechanism resides in the body wall. In ligatured worms the alimentary canal is eliminated, and in the cylinder preparations neither the alimentary canal nor the reproductive system is available as a surface for exchange.

SUMMARY

1. The total osmotic pressure, electrical conductivity and chloride concentration of the body fluid of *Ascaris lumbricoides* and of the intestinal contents of the pig have been measured.

2. The results obtained agree with the observations of previous workers that *Ascaris* normally lives in a hypertonic medium and that it swells or shrinks in saline media which are too dilute or too concentrated.

3. Experiments comparing the behaviour of normal and ligatured animals show that both the body wall and the wall of the alimentary canal are surfaces through which water can pass.

4. 30% sea water has been used as a balanced saline medium for keeping the worms alive in the laboratory. This concentration was selected as being the one in which there was least change in the body weight of the animals exposed to it.

5. The osmotic pressure of the body fluid of worms kept in 30% sea water is approximately the same as in animals taken directly from the pig's intestine. The body fluid of fresh worms is hypertonic to 30% sea water and hypotonic to the intestinal fluid. In 30% sea water the normal osmotic gradient across the body wall is therefore reversed.

6. In 30% sea water the total ionic concentration (as measured by the conductivity) decreases slightly, but the chloride concentration increases by about 50%, although still remaining much below that of the external medium.

7. Experiments in which the animals were allowed to come into equilibrium with various concentrations of sea water from 20 to 40% show that there are corresponding changes in the osmotic pressure of the body fluid which is, however, always slightly above that of the saline medium. The conductivity also changes in a similar manner but is always less than that of the medium, and the difference between the two becomes progressively greater the more concentrated the medium.

8. The chloride concentration of the body fluid varies with but is always below that of the external medium, whether this is intestinal fluid or one of the saline media. In the latter the difference between the internal and external chloride concentrations is least in 20% sea water and becomes progressively greater as the concentration of the medium is increased.

9. Experiments with ligatured worms and with eviscerated cylinders of the body wall show that these share the capacity of the normal worm to maintain the chloride concentration of the body fluid below that of the environment. This power is not possessed by cylinders composed of the cuticle alone.

10. If the worms which have had their internal chloride concentration raised by exposure to 30% sea water are transferred to a medium composed of equal volumes of 30% sea water and isotonic sodium nitrate solution, the chloride concentration of the body fluid is reduced to a value below that of the external medium. This phenomenon is also displayed by worms ligatured after removal from the 30% sea water and, to an even more marked degree, by eviscerated cylinders of the body wall.

11. It is concluded that *Ascaris* is able to maintain the chloride concentration of the body fluid below that of the external medium by an active process of chloride excretion against a concentration gradient, and that this mechanism is resident in the body wall, the cuticle being freely permeable to chloride.

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APPENDIX

The Freezing-Point Method of Determining Osmotic Pressures

By W. Stephenson

Introduction

The method described below is essentially similar to that employed by hydrocarbon chemists (Mair, Glasgow & Rossini, 1941) and metallurgists (Stockdale, 1930). No description of the method seems to be available in biological literature, and it does not appear to have been generally used in this field. The method had the advantage of avoiding the complicated apparatus and technique associated with many of the methods previously described (Johlin, 1931; Schopfer, 1932; Stadie & Sunderman, 1931), while it yields results of comparable accuracy.

Method

The normal Beckman freezing-point apparatus is used, cooling is effected by a mixture of one part of common salt with three of ice, contained in a Dewar flask. This is separated from a boiling tube containing the experimental liquid by means

of an air jacket. The hole in the boiling tube cork which admits a glass stirrer is sufficiently large to allow glass beads to be dropped into the liquid with the stirrer in position. The glass beads are contained in a separate tube let into the freezing mixture, and are surrounded by a thin film of frozen experimental liquid.

The zero point on the thermometer is found daily in the usual way, and following this, about 20 ml. of the completely thawed experimental fluid is introduced to the apparatus. By sliding the tube containing the experimental liquid into or out of the air jacket, the rate of cooling of the liquid is adjusted to about 0·25° C. per ½ min. at the approximate freezing-point. After this adjustment, and until the end of the determination, temperatures are noted at half-minute intervals to the nearest 0·002° C. It is essential that stirring should be thorough, regular and continuous throughout. When the experimental liquid is supercooled between 0·2 and 0·4° C., a 'frozen' glass bead is introduced via the hole in the cork. The liquid begins to freeze immediately, and temperatures rise to an approximately steady value before slowly falling again. Temperatures are noted for approximately 12 min. of this period of slow fall. The boiling tube and contents are then removed, the fluid thawed to about 1·5° above its freezing-point, and the determination repeated. Further repetitions are sometimes necessary.

Treatment of results

Results are plotted as time-temperature curves, an actual example being shown in Fig. 3. In this figure, *XY* represents the cooling curve of the unfrozen liquid, *ZA* the rise in temperature at the beginning of the freezing, *A* the 'plateau' value and *BC* the slow decrease in temperature after the plateau, during which progressive freezing of the liquid occurs.

Mair *et al.* (1941) have shown that if the later portion of the cooling curve following supercooling (*BC* of Fig. 3) is extrapolated backwards, the resulting curve is approximately the cooling curve which would be obtained if supercooling had not occurred. The intersection of this curve (*CB*) with the cooling curve of the unfrozen liquid (*XY*) thus gives the approximate freezing-point of the solution (*A'*). As the supercooled liquid is nearer the jacket temperature than the equivalent portions of the extrapolated curve, it will cool at a slightly slower rate. To allow for this, a small correction should be applied to the freezing-point obtained. This correction is negligible if the amount of supercooling is small compared with the difference between the freezing-point and the jacket temperatures, or if supercooling is of short duration. These conditions are fulfilled by following the procedure described above.

As a practical detail, care must be taken in these extrapolations to neglect the points immediately following the plateau in the cooling curve (i.e. not to extrapolate along the curve *CBA*), since at this stage the after-effects of supercooling have not reached completion.

Accuracy of results

When consecutive determinations were made upon an inorganic solution (30 % sea water) it was noted that the second determination generally gave smaller freezing-point depressions than the first. The average reduction with six pairs of determinations

was 0·0020° C. This is probably due primarily to changes in the volume of the thermometer glassware upon cooling, and if so could be minimized by storing the

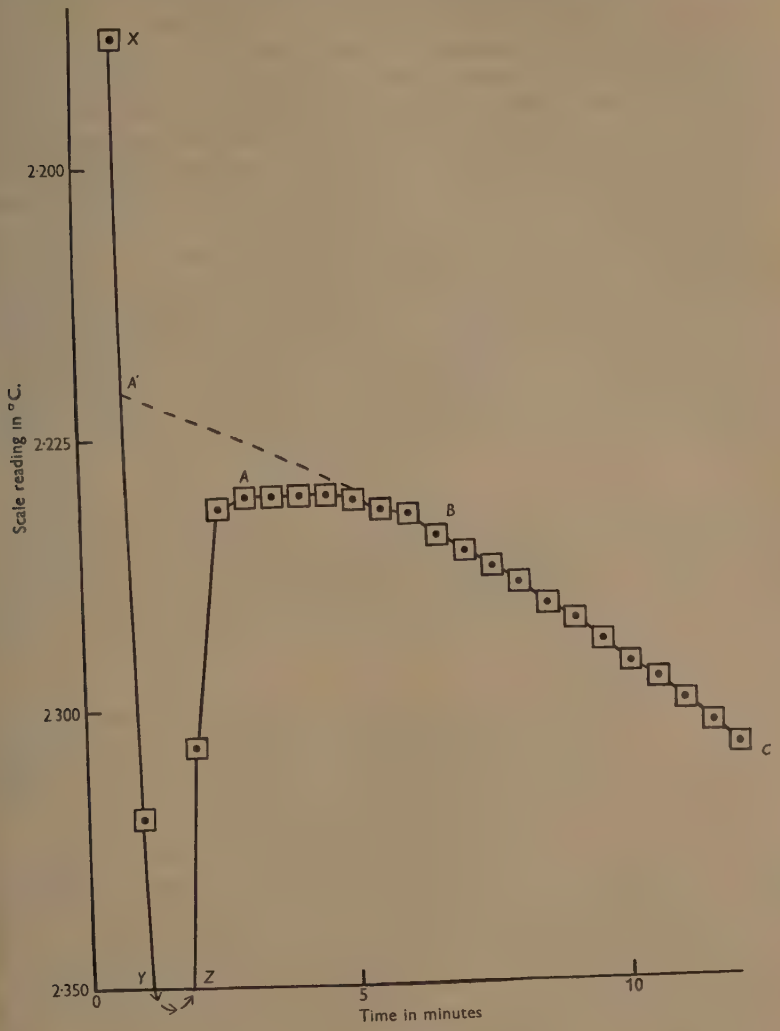

Fig. 3. For explanation see text.

thermometer at about 0° C. In addition it is possible that the solution is diluted upon cooling by condensation of moisture from the air.

When *Ascaris* body fluid and intestinal fluid from the pig were used, somewhat

similar tendencies were observed. Here the reductions in freezing-point depressions were: between first and second determinations, 0·0077° C. (mean of 31 pairs of results); subsequent determinations, 0·0029° C. (mean of 37 pairs of results). The second of these values is sufficiently close to that obtained with diluted sea water (0·0029° C.) as to suggest that similar causes are operating. The first value (0·0077° C.) is so much larger that it suggests a different cause. It is possible that the first freezing of the above biological fluids causes the precipitation to colloidal material, with a corresponding reduction in osmotic pressure. Most of the samples had been kept previously in a refrigerator at about −1° C., and it was observed that a fine yellow precipitate had settled from the *Ascaris* body fluid. The final values of the depressions of the freezing-point which are obtained are therefore not likely to give a true absolute value of the osmotic pressure of the fresh material.

To obtain comparatively stable values for the depression of the freezing-point, determinations were continued until the last pair of values agreed to within the nearest 0·010°. An average difference of 0·0041° was thus obtained between the final pairs of results for 37 different liquids, and the mean of each pair will thus be accurate to about the nearest 0·003°. Converted to mmol. NaCl by data given in Hodgman (1936, p. 1297), this means that the results are accurate to about 1 mmol. NaCl.

Discussion

Early workers in the biological field, such as Atkins (1909), determined depressions of the freezing-point by direct measurements of the plateau temperature obtained after supercooling. Before and since then this method has been shown to be unsound, and the formulae of Raoult (1898) show that this method can only give reliable results if the amount of supercooling and the rate of cooling after the attainment of the plateau are negligible.

Raoult's principle of applying calculated corrections to the observed plateau values was used with success by the earlier physical chemists. It can be shown that for this success it is essential that, after the attainment of the plateau, the convergence temperature (i.e. temperature the experimental liquid would attain at infinite time) should be very close to the true freezing-point of the liquid.

The most satisfactory method so far described for use with biological material is due to Johlin (1931). In this method the experimental liquid is rapidly cooled to just below its freezing-point, and the temperature then maintained at this level. The liquid is seeded with metal rings cooled in solid carbon dioxide, and on freezing there is a slight rise in temperature to a stable plateau value which needs no correction. In this method it is necessary to alter the temperature of the air jacket during the experiment. This is accompanied by the use of an alcohol and solid carbon dioxide freezing mixture, and by altering its level relative to that of the experimental liquid. Schopfer (1932) used a somewhat similar principle, and obtained different rates of cooling by altering the rate at which dry air was pumped through ether. Both these methods involve an elaboration of the apparatus and experimental technique. These disadvantages are absent from the extrapolation method described

above. Although large quantities of liquid were used in the present work, the method can easily be adopted for semi-micro work by the employment of a micro-Beckmann thermometer.

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STUDIES ON THE PHYSIOLOGY OF *ASCARIS LUMBRICOIDES*

II. THE INORGANIC COMPOSITION OF THE BODY FLUID IN RELATION TO THAT OF THE ENVIRONMENT

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For the investigation of many aspects of the physiology of *Ascaris lumbricoides* and other parasitic nematodes the provision of a fluid medium in which they can be kept alive and healthy in the laboratory is necessary. This is a matter of considerable difficulty, since the natural environment of such animals contains organic substances which are subject to bacterial action. Consequently, most of the experimental work which has been carried out has been on animals kept in saline solutions to which organic constituents were not added. In view of the fact that balanced salt solutions are usually employed in physiological work, it is somewhat surprising to find that solutions of sodium chloride have been used by many recent workers (see, for example, Harnisch, 1933, 1935; Hoffmann, 1934; Waechter, 1934; Krüger, 1936, etc.). Dewitz (1899), however, used sea water in the case of an unidentified nematode from the tissues of *Scomber scombrus*. Some later workers, such as Slater (1925), Adam (1932) and Hirsch & Bretschneider (1937), used Ringer or Tyrode's solution for *Ascaris lumbricoides* and Toryu (1934) kept *Parascaris equorum* in Ringer. Davey (1938), working with adult *Ostertagia circumcincta* and Fenwick (1939) with larvae of *Ascaris lumbricoides*, developed, by means of a series of survival experiments, saline media in which the animals could be kept alive in the laboratory for some days. Baldwin (1943) also used a balanced salt solution in which adult *A. lumbricoides* would survive for as long as 10–11 days, although the worms were undoubtedly unhealthy after the first few days in this medium. Hobson, Stephenson & Beadle (1951; and see Hobson, 1948), working with *A. lumbricoides*, in 1941 used 29% and, later, 30% sea water as a medium. In this, individual specimens have survived in apparently healthy condition for as long as 28 days. Baldwin & Moyle (1947) have used a medium based upon Eden's analyses (see later in the present paper) of the body fluid of the same species. In this the animals did not live longer than in Baldwin's original medium (Baldwin, 1943), although they remained more active. The composition of the various media to which reference has been made is given in Table 1.

The observations of Hobson *et al.* (1951) show clearly that the body fluid of *A. lumbricoides* changes appreciably in composition when the animals are exposed to a purely saline medium such as 30% sea water, and that its chloride content is considerably increased. Although no artificial medium lacking organic constituents

Table 1

Medium	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)	Cl (mmol.)	SO_4 (mmol.)
Davey's saline	154	6	3	Nil	166	Nil
Fenwick's saline	137	3	2	2	148	Nil
Baldwin's saline	136	3	2	0·4	141	0·4
Baldwin & Moyle's saline	130	24	6	5	170	Nil
30% sea water	141	3	3	16	164	8·5

corresponding to those occurring in the normal environment can be quite satisfactory, it is clearly desirable that its electrolyte composition should correspond as closely as possible to that of the intestinal fluid of the host. Further, the relation of the composition of the external medium to that of the body fluid of the worm should be considered.

For this reason analyses have been made of the principal inorganic constituents of the intestinal fluid of the pig, of some artificial media, of the body fluid of fresh worms and of worms which have been exposed to the saline media. These were carried out by A. Eden at the Ministry of Agriculture and Fisheries' Veterinary Laboratory, Weybridge. W. Stephenson and A. D. Hobson were responsible for the conductivity and osmotic pressure determinations as well as for some of the measurements of chloride concentration.

ANALYTICAL METHODS

The samples of the various body fluids were taken to Newcastle in test-tubes, stoppered with rubber bungs, and packed in large thermos flasks containing cotton-wool and broken ice. They were dispatched by passenger train to Weybridge, and the temperature of the samples on receipt was always below 5° C. Each sample was warmed to 20° C., and a known volume (5 or 10 ml.) was weighed into a silica basin to obtain the specific gravity. Most of the moisture was then carefully evaporated off by placing the basin on a hot plate, and the residue was then dried to constant weight in an oven at 105° C. The basin was ignited gently, first over a small flame and then in a muffle furnace at 500° C., until all carbonaceous matter had burned away, giving the total ash figure.

The ash was taken up carefully with dilute HCl, evaporated to dryness, and dissolved in hot water in the presence of 1 ml. decinormal HCl. The resultant solution was washed into a 200 ml. flask, and the transference completed by hot water; after cooling the contents of the flask were made up to the mark and thoroughly mixed. This solution was used for the determination of calcium, magnesium, sodium and potassium.

Calcium was determined on a suitable aliquot (1, 2 or 5 ml.) by the Clark-Collip (1925) modification of the Kramer-Tisdall procedure, being precipitated as oxalate, which was determined by acid-$KMnO_4$ titration. The supernatant fluid of the original precipitation, obtained by centrifuging the oxalate precipitate, was used for the estimation of *magnesium*, which was precipitated as magnesium ammonium

phosphate and the latter determined by the molybdenum blue procedure of Fiske & Subba Row (1925) for inorganic phosphate. *Sodium* was determined gravimetrically by precipitation in a sintered glass crucible with zinc uranyl acetate, after removal of phosphates with solid calcium hydroxide. *Potassium* was determined on suitable aliquots by sodium cobaltinitrite precipitation by a modification of the Jacobs-Hoffman procedure (Eden, 1943).

Phosphorus was determined separately on 2 ml. portions of the original body fluid by wet digestion to remove organic matter employing sulphuric, nitric and perchloric acids, dilution to a known volume, and developing the molybdenum blue complex by the method of Fiske & Subba Row (1925). This procedure gave a total phosphorus figure, since it was felt that inorganic phosphorus determination might give unreliable results, owing to the uncertain effect of any phosphatases that might be present. *Chlorides* were also determined on the original fluid by a minor modification of the Whitehorn method (1921) for blood.

ELECTROLYTE COMPOSITION OF THE NORMAL ENVIRONMENT

A. lumbricoides normally inhabits the small intestine of its host. In the investigations described here the animals were obtained from the pig. In Table 2 are given the results of analyses of a number of samples of the intestinal contents of each pig. Each sample was composed of approximately equal volumes of fluid obtained by centrifuging material obtained from the small intestine of a number of pigs immediately after slaughtering.

Table 2. *Composition of the contents of the small intestine of the pig*

Sample	Date of sample	No. of pigs	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)	Cl (mmol.)	P (mmol.)	Dry matter (%)	T s (
A	15. ix. 42	11	277	47·6	5·7	11·1	83	26·8	6·84	
B	15. ix. 42	11	277	46·0	5·5	14·0	73	26·8	6·70	
Av. samples A and B			277	46·8	5·6	12·6	78	26·8	6·77	
31	5. i. 43	5	117	20·7	27·4	6·6	42	22·9	5·4	
32	5. i. 43	5	119	22·2	7·2	7·0	38	23·2	5·6	
33	5. i. 43	5	117	26·6	6·5	5·8	51	22·6	5·8	
34	5. i. 43	5	137	35·0	2·2	4·1	88	26·4	7·8	
35	5. i. 43	5	121	21·5	36·9	6·2	60	23·9	6·3	
36	5. i. 43	5	134	34·0	2·0	3·3	88	26·4	7·8	
Av. samples 31–36			124	26·7	13·5	5·5	61	24·2	6·5	

It will be noted that the samples fall into two groups which differ considerably in their electrolyte composition. Samples A and B, taken on the same day, are characterized by the fact that their sodium, potassium and magnesium contents are about twice as great as those of samples 31–36, also taken on one day. The volumes for calcium, on the other hand, are of the same order of magnitude in both sets if we except the extraordinarily high concentrations in samples 31 and 35. It should be noted that the values given for phosphorus do not distinguish between inorganic and organic phosphorus.

Table 3. *Analyses of pooled samples of body fluid of* Ascaris lumbricoides *obtained fresh from the host without exposure to an artificial medium*

Date	No. of animals in sample	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)	Cl (mmol.)	Total P (mmol.)	Specific gravity at 20° C.	Dry weight (%)	Total ash (%)	Conductivity ≡ mmol. NaCl	Osmotic pressure as ≡ mmol. NaCl
26. v. 42	50	118	36·6	9·0	4·9	46·2	19·0	1·016	6·26	0·85	137	187
2. vi. 42	50	134	16·4	5·0	4·5	56·4	16·1	1·018	6·52	0·90	144	205
7. vii. 42	(46)	130	21·5	5·5	4·9	51·3	18·7	1·015	6·76	0·90	139	198
7. vii. 42	27	123	26·1	5·7	5·8	54·7	17·1	1·018	6·73	0·87	145	196
14. vii. 42	39	133	19·0	4·7	4·5	56·9	14·6	1·016	6·48	0·94	140	196
16. xii. 42	70	126	30·5	5·5	4·9	51·2	16·8	1·019	7·60	1·00	147	200
16. xii. 42	70	138	22·0	6·0	4·9	53·1	16·8	1·017	7·40	0·90	148	206
Mean (unweighted)	—	129	24·6	5·9	4·9	52·7	17·0	1·017	6·82	0·91	143	198
Rogers* (1945)	—	119	—	—	—	55·1	12·0	—	6·64	—	—	—

* Averages calculated from Table 1 of Rogers's paper.

The analyses quoted show that there is, as might perhaps be expected, a great variation in the electrolyte composition of the medium to which the worms are normally exposed. The causes of these differences cannot be determined without detailed knowledge of the previous history of the pigs from which the material was obtained. This was not available.

ELECTROLYTE COMPOSITION OF THE BODY FLUID OF *ASCARIS LUMBRICOIDES* OBTAINED DIRECTLY FROM THE HOST

Little has been published on the composition of the body fluid of nematode worms. Flury (1912), Schopfer (1924, 1926) and Duval & Courtois (1928) give values for the chloride concentration in *Parascaris equorum*. Rogers (1945) made determinations of certain constituents of the body fluid of *Ascaris lumbricoides*, and average values calculated from his figures are quoted at the foot of Table 2. He also gives data for glucose and for protein and non-protein nitrogen.

In Table 3 are given the results of the analyses of seven pooled samples of the body fluid of varying numbers of worms which had been taken direct from the host and had not been exposed to any artificial saline medium. These show that the electrolyte composition of the body fluid is fairly constant, especially in its calcium and magnesium content. At the risk of being accused of selecting figures it may be permissible to compare the mean values for the body fluid with those for samples 31–36 of the intestinal fluid of the pig, leaving out the calcium figures for samples 31 and 35, which are obviously abnormal. This comparison is given in Table 4.

Table 4

	Na	K	Ca	Mg	Cl	P
Intestinal fluid of pig	124	26·7	4·5	5·5	61	24·2
Body fluid of *A. lumbricoides*	129	24·6	5·9	4·9	53	17·0

From these figures it appears that the electrolyte composition of the body fluid of *A. lumbricoides* is closely similar to that of its normal environment, with the exceptions that the phosphorus and chloride concentrations are lower. The problem of the chloride concentration has been discussed in an earlier paper (Hobson *et al.* 1951).

THE RELATION OF THE ELECTROLYTE COMPOSITION OF THE BODY FLUID OF *ASCARIS LUMBRICOIDES* TO THAT OF THE ENVIRONMENT IN ANIMALS EXPOSED TO A SALINE MEDIUM

In earlier work (Hobson *et al.* 1951) 30% sea water was used as a balanced saline medium for keeping animals alive in the laboratory. This proved satisfactory for short-term experiments, and individuals have been kept for as long as 28 days alive and healthy as far as could be judged by external appearance and activity. It seemed, however, important to discover what changes occur in the electrolyte composition of the body fluid of worms exposed to this and other saline media. The values after various times of exposure are given in Table 5.

Table 5. *Analysis of saline media and of pooled samples of the body fluid of* Ascaris lumbricoides *exposed to the media*

Date	Material	No. of worms used for sample	Na (mmol.)	K (mmol.)	Ca (mmol.)	Mg (mmol.)	Cl (mmol.)	P (mmol.)	Specific gravity at 20° C.	Dry weight (%)	Total ash (%)	Conductivity ≡ mmol. NaCl	Osmotic pressure ≡ mmol. NaCl	Time animals exposed to medium
	30 % sea water	—	141	3	3	16	164	—	—	—	—	184	176	—
6. viii. 42	Body fluid of worms in 30 % sea water	33	133	11·5	4·7	4·9	70·5	13·2	1·015	6·20	0·92	131	195	44
29. viii. 42	Body fluid of worms in 30 % sea water	35	125	16·6	4·2	4·9	82·9	12·6	1·013	5·57	0·88	133	187	87
15. viii. 42	Body fluid of worms in 30 % sea water	38	120	12·3	3·7	5·3	79·0	10·3	1·012	4·57	0·79	126	176	96
	Artificial saline medium A	—	126	20·2	19·7	3·3	162·4	2·4	1·002	1·1	1·0	170	171	—
28. v. 43	Body fluid of worms in artificial media A	40	128	10·7	4·7	4·5	78·1	12·9	1·008	5·4	0·8	133	178	48
28. v. 43	Body fluid of worms in artificial media A	53	114	10·0	4·2	4·1	73·9	12·6	1·003	5·2	0·8	134	186	48
28. v. 43	Body fluid of worms in artificial media A	44	123	11·3	3·7	3·7	80·1	10·3	1·015	4·7	0·9	130	181	96
30. vi. 43	Artificial saline medium B	?	120	13·6	4·0	4·1	75·9	10·3	1·013	4·6	0·8	132	183	96
	Body fluid of worms in artificial media B	—	137	38·9	3·2	9·5	200·0	2·4	1·004	1·4	1·3	208	196	—
	Body fluid of worms in artificial media B	14	148	15·6	5·0	5·8	80*	13·9	1·004	5·8	1·0	151	206	48
	Body fluid of worms in artificial media B	17	149	15·3	4·2	5·8	76*	13·5	1·002	6·0	1·0	151	208	48
	Body fluid of worms in artificial media B	18	140	19·2	3·7	5·3	88*	12·9	1·013	5·6	1·0	153	209	96
	Body fluid of worms in artificial media B	21	148	18·2	4·0	5·3	77*	11·6	1·011	5·5	1·0	155	210	96

* Chlorides determined by Hobson *et al.*

The results may be summarized as follows:

Sodium. The sodium concentrations of the three saline media used did not differ greatly from those of the intestinal fluid of the pig as shown by the analyses of samples 31–36 in Table 2. The amount of sodium in samples A and B seems to be abnormally high. The sodium concentration of the body fluid of *Ascaris*, although somewhat variable, is of the same order of magnitude in fresh animals and in those which have been exposed to saline media. The data presented do not indicate that the concentration of sodium in the body fluid of the animals varies with that of the external medium. It must be remembered, however, that the range of concentrations in these experiments was small.

Potassium. Comparison of the values given in Tables 2 and 3 suggests that the concentration of potassium in the body fluid of fresh *Ascaris*, although variable, is approximately the same as that of its normal environment. (The potassium content of samples A and B of intestinal fluid seems, like that of the sodium, to be abnormally high.) When the animals are transferred to a saline medium there is a marked fall in the potassium concentration of the body fluid. Moreover, the internal concentration does not show any clear correspondence with that of the external medium.

Calcium and magnesium. The concentrations of these two elements in the body fluid of *Ascaris* remain very constant, and there is no significant difference between the values obtained with fresh worms and those which have been exposed to artificial media.

Chloride. In an earlier paper (Hobson *et al.* 1951) it has been shown that the chloride concentration of the body fluid varies with but always remains below that of the external medium. This conclusion receives further support from the data presented here.

DISCUSSION AND SUMMARY

The results obtained in this investigation are admittedly not as extensive as is desirable but they allow certain conclusions to be drawn.

1. The sodium and potassium contents of the body fluid of *Ascaris lumbricoides* are somewhat variable, but these variations do not seem to be dependent upon those of the external medium.

2. The calcium and magnesium contents of the body fluid are relatively constant and are not affected by those of the external medium.

3. The chloride concentration of the body fluid is closely related to and always remains lower than that of the external medium.

4. As shown in Table 2, there is a large gap between the total concentrations of inorganic cations and anions in the intestinal fluid of the pig. Presumably a considerable proportion of the inorganic cations are combined with organic anions, at present undetermined. Exposing the worms to saline media composed of chloride caused a large rise in the internal chloride concentration. This may well be a limiting factor in the life of the animals in such media, and the next step forward would seem to be the fuller analysis of the environment to which they are normally exposed.

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AN ELECTRON DIFFRACTION STUDY OF THE STRUCTURE AND ORIENTATION OF THE LIPIDS IN YEAST AND BACTERIAL CELL WALLS

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(With Plates 1–4 and Three Text-figures)

While it is generally agreed that many of the permeability relationships of different types of living cells depend on the chemical and physical properties of the lipids or lipo-proteins in the surface layers, no direct evidence of the nature of the molecular organization of these fatty components as they occur *in situ* has yet been produced. Such suggestions as have been made have been based mainly on permeability studies of cells and model membranes, while examinations of the properties and behaviour of the isolated components by the sensitive monolayer technique (Schulman & Rideal, 1937) has proved a useful guide to the possible molecular structure of the cell membrane. Supporting evidence has been derived from polarization and electron microscopy, and from impedance and electrophoresis measurements. The thinness of the cell membrane has so far prevented direct study by X-ray diffraction, although this method of analysis has successfully been applied to the study of isolated fatty components (Schmitt & Palmer, 1940).

Electron diffraction methods have proved of considerable value in the study of fatty films which may be only a few molecules thick (Germer & Storks, 1938; Schoon, 1938; Coumoulos & Rideal, 1941; Brummage, 1947; Hurst, 1950); and since the cell membrane has been supposed to include oriented lipids, it seemed possible that the same methods might be sufficiently sensitive to reveal the structure of the lipids in the membrane. Yeast and bacterial cells were used to test this possibility, because the material was readily available; because changes in shape and structure of the cells could be studied by electron microscopy; and because preliminary studies had shown this material to be eminently suitable for work on permeability and cytoplasmic structure. (An account of this last aspect of the work will be given in subsequent papers.)

Little is known of the detailed structure or composition of the surface membranes in yeasts and bacteria. In these cells, the membrane at the surface of the cytoplasm is rich in lipids (Knaysi, 1938) and ribonucleic acid (Bartholomew & Umbreit, 1944; Henry & Stacey, 1946; Bartholomew & Mittwer, 1951). This membrane secretes an outer cell wall, which contains complex polysaccharide materials and is responsible for the shape of the cell (Salle, 1943; Dubos, 1949; Bisset, 1950; Clifton, 1950). There is only indirect evidence for the presence of proteins or lipo-proteins in the cell wall; but it has been suggested that the action of surface-active detergents in modifying the structure and permeability of the cell wall may be due to intereaction

with the lipids, proteins, or lipo-proteins present (Baker, Harrison & Miller, 1941; Valko, 1946; Davis & Dubos, 1947; Dubos, 1949; Kodicek, 1949). In the present work, fat solvents have been used to displace lipids from the framework of the cell wall, and differences between the structure and orientation of the fat-soluble lipids and bound lipids in this membrane have been studied by electron diffraction methods and by light and electron microscopy.

MATERIAL AND METHODS

Since electron diffraction and electron microscopy methods involve the use of dried material, a direct indication of the nature and degree of cell distortion during dehydration was obtained by light microscopy, using yeast cells as test material. Changes in the structure of the hydrated cells in the presence of fat solvents were also observed by this method.

Cell preparations

Pure cultures of bakers' yeast (*Saccharomyces cerevisiae*), and of *Escherichia coli*, were grown on 5% malt-agar slopes; *Staphylococcus aureus* was grown on 5% nutrient agar slopes. All cultures were incubated at 30° C., and cell suspensions were prepared by flooding the surfaces of the slopes with sterile distilled water. Stock suspensions were freshly prepared in this way for each particular group of experiments. Fresh commercial bakers' yeast was used for studying the effects of fat solvents on the structure of the hydrated yeast cell, and on cell shape after dehydration.

Fat solvents

It has been shown that when *Calliphora* pupal skin is treated with chloroform, the fat-soluble lipids are displaced (Hurst, 1950). The diffraction pattern of the residual, or bound lipids is weaker than that of the untreated cuticle, since the displacement of lipid results in a reduction in the number of electron-scattering points in the cuticle. The bound lipids, however, are thermo-stable, and the pattern does not fade when the cuticle is exposed to a more intense electron beam. Under similar conditions, the pattern of the untreated cuticle fades, owing to thermal disorientation of the fat-soluble crystals of waterproofing wax. It was therefore suggested that the bound lipids are stabilized by their association with structural proteins of the epicuticle (Hurst, 1950).

In order to ascertain whether a similar distinction between the bound and fat-soluble lipids could be made in yeast and bacterial cells, cell suspensions were treated with fat solvents, the lipid-dispersant activities of which were known in relation to the insect epicuticle (Hurst, 1943, 1948). Samples (2 c.c.) of a given cell suspension were introduced into small tubes, and fat solvents were added to give the following concentrations in the suspensions: (*a*) hexane (5%); (*b*) ether (10%); (*c*) chloroform (2·5%); (*d*) ethyl alcohol (30%); (*e*) propyl alcohol (15%); (*f*) butyl alcohol (9%); (*g*) amyl alcohol (3%); (*h*) ethyl alcohol (30%)–ether (10%). Where other concentrations were used, these are referred to in the text. The further treatment of the cell suspensions will be indicated subsequently.

(*a*) *Examination of hydrated cells* (*light microscopy*)

One part of commercial bakers' yeast was suspended in twenty parts of distilled water. Samples of suspension were treated with fat solvents in the manner described, and the tubes were shaken vigorously to disperse the solvents in the suspensions. The concentrations of ether, butyl alcohol, amyl alcohol and chloroform used were sufficient to give saturated solutions in the suspensions at room temperature (*c.* 20° C.). Hexane formed a relatively unstable emulsion. By means of a glass rod, drops of the suspensions were transferred to glass slides, covered with thin cover-slips and examined at high magnification (× 1000) with a microscope, using a $\frac{1}{12}$th oil-immersion objective. The preparations were strongly illuminated from below, and micro-photographs taken of suitable preparations to illustrate changes in cell shape and structure.

Observations were also made on the changes in cell distribution which occurred when thin aqueous layers of suspension deposited on slides when the water was allowed to evaporate in the absence of cover-slips.

(*b*) *Examination of dried cells* (*light microscopy*)

Nigrosin 'shadowing' technique. Preliminary observations showed that it is difficult to distinguish clearly the shapes of dried yeast cells deposited from an aqueous solution on glass slides. If the aqueous suspension contains a fairly strong solution (1 %) of nigrosin, however, the transparent cells are more readily seen in aqueous suspension owing to the negative staining effect of the dye.

When a layer of the suspension is allowed to evaporate on a glass slide before examination, the watery phase gradually recedes from the cell surfaces, and a deposit of dye is left on the exposed surfaces. At the same time, the evaporation of the water causes an increase in concentration of dye in the residual water layer so that the intensity of staining increases with decrease in thickness of the layer. This results in a 'shadowing' of the peripheral regions of the cell when viewed by transmitted light, and a three-dimensional impression of the shape of the cell is obtained. If the cells are flattened, the dye is deposited more uniformly on the flattened surfaces, and the 'shadowing' effect observed with spherical or oval cells is less marked.

Nigrosin was added to a suspension of yeast cells in water (one part yeast to twenty parts distilled water) to give a 1 % concentration of dye in the suspension. This was divided into 2 c.c. samples in small tubes, and fat solvents were added and dispersed as already described. Drops of suspension were spread on glass slides and allowed to evaporate. The dried deposits were covered by cover-slips, secured to the slides by vaseline applied to the edges of the slips, and examined with a $\frac{1}{12}$th oil-immersion objective.

Side-view appearance of dried cells. The edges of square glass cover-slips (no. 2) were rounded by passing them slowly through a bunsen flame. Drops of yeast suspension, prepared as for use in the nigrosin 'shadowing' technique, were applied to the rounded edges and adjacent surfaces of the slips, which were rotated so that the deposits dried uniformly. The slips were placed on glass slides and covered with

larger slips secured at the edges to the slides by means of vaseline. The preparations were then examined with the oil-immersion objective, focusing on the extreme outer edge where the glass surface was approximately parallel to the microscope tube. This restricted region of the curved edge appeared as a sharp outline in optical section. Any cells or groups of cells located in this restricted region were then seen from the side in optical section. The appearance of the dried cells was compared with those examined by the nigrosin 'shadowing' technique.

(*c*) *Examination of dried cells* (*electron microscopy*)

Cell suspensions containing added fat solvents were shaken at frequent intervals over a period of 20 min. at room temperature, and then washed by centrifuging three times with distilled water. After suitable dilution in water, drops of suspension were transferred by means of a platinum loop to collodion bases mounted on the standard circular grids used with the R.C.A. electron microscope and diffraction unit. The dilutions chosen and the sizes of drops applied to the grids were adjusted to give a single layer of cells, covering about 50% of the surface of each collodion base after this had dried in a desiccator over calcium chloride. Residual traces of water were removed in the electron microscope. The dried cells were on the side of the collodion base furthest from the beam.

(*d*) *Examination of dried cells and collodion/lipid membranes* (*electron diffraction*)

Dried cells. After suitable cell preparation had been examined with the electron microscope, the diffraction unit was introduced and the diffraction patterns photographed. The voltage across the electron gun was 50 kV. The beam currents varied from 150 to 250 μA., according to the transparency of the particular preparations. The complete range of fat solvents (*a*)–(*h*) (p. 31) was tested on *Escherichia coli* cells; a restricted range (*a*)–(*c*) was used with yeast and *Staphylococcus aureus*.

Collodion/yeast-lipid membranes. In order to compare the crystalline structure of the cell lipids with that of the isolated fat-soluble lipids from the same cells, a lipid extract was prepared from commercial bakers' yeast, since this material could readily be obtained in sufficient quantity. Fifty grams of moist fresh yeast were stirred continuously with 20 c.c. of warm chloroform (40° C.) for 30 min. in a beaker. The chloroform extract was filtered and evaporated to dryness under reduced pressure, leaving a yellowish brown greasy residue. This was markedly hydrophilic; it spread rapidly on a water surface, and dispersed in water on shaking to give a relatively stable emulsion.

Collodion/yeast-lipid membranes were prepared using the technique which has been described in a previous paper for the preparation of collodion/fatty acid membranes (Hurst, 1950). Yeast-lipid (3%) was dissolved in a 1% solution of collodion in amyl acetate, and a drop of the solution allowed to spread on the surface of water contained in a large dish. After the solvent had completely evaporated, a thin membrane, *c.* 200 A. thick, was left floating on the water surface. Portions of the membrane were mounted on grids similar to those used for the examination of the

yeast and bacterial cells. The preparations were dried in a desiccator over calcium chloride before they were introduced into the electron microscope.

Collodion/phospholipid membranes. Since lecithin and kephalin are typical cell lipids, similar in physical properties to the isolated yeast lipids, their crystalline structure was also examined. Membranes were prepared by spreading collodion solutions containing 3% phospholipid on a water surface, the technique being similar to that used for the yeast-lipid extract. Purified samples of lecithin (Hammarsten) and kephalin (Blix) were used.

The side-spacings corresponding to the rings in the diffraction patterns were calculated by comparison with the rings in the diffraction transmission pattern of micro-crystalline gold film deposited on a collodion base. The method of calculation has been described in a previous paper (Hurst, 1950). Differences in thermal stability between the lipids in the cells and in the collodion membranes were used as criteria for distinguishing between the fat-soluble and bound lipids of the cell wall.

RESULTS

I. *Observations on hydrated yeast cells before treatment with fat solvents*

In aqueous suspension the yeast cells are spherical or oval in shape. The cell wall cannot easily be distinguished from the inner transparent cytoplasm, which contains the characteristic large 'sap vacuole' and a few conspicuous granules (Henry & Stacey, 1946; Clifton, 1950). When a strong sugar solution is added to a yeast suspension on a slide, the cells are plasmolysed, and the cytoplasm retracts from the outer translucent cell wall. The plasmolysed cells as a whole, however, are smaller than the normal cells, and less regular in shape.

Adsorption of cells at air-water interface. It was observed that where the thickness of the water layer between the cover-slip and slide was sufficient to permit free movement of yeast cells, these did not aggregate or clump together, but were more or less uniformly distributed in the bulk water layer. At the air-water interface, however, where the water was evaporating, the cells were adsorbed and tended to form a continuous layer as the surface area of this interface decreased owing to evaporation. The cells in this continuous layer became compressed so that the average width was less than that of cells in the adjacent water phase (Pl. 1, fig. 1).

Effect of mechanical pressure on cells. Pressure was exerted on the cells in an aqueous yeast preparation by means of two small spring clips applied to the opposite edges of the coverslip. It was noted that under these conditions many of the cells were motionless, suggesting that they were held fast between the slide and cover-slip. When the spring clips were removed, the cells once again moved freely in the watery layer, and were apparently undamaged.

II. *Effects of fat solvents on structure of hydrated yeast cells*

Hexane. Hexane dispersed on shaking in the aqueous suspension of yeast cells to give a coarse unstable emulsion of which droplets of hexane tended to coalesce, so that extensive hexane-water interfaces of considerable area formed. When yeast cells moved from the bulk water phase into contact with a hexane-water interface,

the cells tended to adhere to the interface, along which they moved, showing that they were attracted to this interface (Pl. 1, fig. 2). The cells became less mobile as the interface became crowded, and in some cases the cells were distorted by lateral compression, as observed with cells adsorbed at an air-water interface (Pl. 1, fig. 1).

In the bulk water phase, the cells were similar in appearance to those in the control aqueous suspension before treatment with hexane. No irreversible changes in cell structure were observed when the cells were subjected to mechanical pressure in the manner described for the control.

Ether. In the presence of ether, the yeast cells were slightly distorted, but in general they were similar in appearance to the controls, although in a few cells the cytoplasm was retracted from the cell wall. The cells were undamaged after mechanical pressure even though they had been in contact with the ether in the tube for an hour before a sample was spread on a slide, and staining of the cytoplasm by nigrosin was slight (Pl. 1, fig. 3).

Chloroform. After treatment with chloroform, the cells were more distorted than by ether treatment, and the cytoplasm was distinctly granular; the large vacuole characteristic of the controls was absent. Mechanical pressure now ruptured the cell wall in many cells, so that the cytoplasm was extruded. Where this occurred the extruded portion of the cytoplasm became progressively more heavily stained by nigrosin than the cytoplasm still surrounded by the cell wall. It was observed, however, that even when the cell wall was apparently intact, the cytoplasm was darker than in the controls or in cells which had been in contact with hexane or ether, suggesting that the penetration of the dye into the cell was facilitated by chloroform more than by these other solvents (Pl. 1, fig. 4).

Ethyl, propyl, butyl and amyl alcohol. At the concentrations used the effects of ethyl and propyl alcohol were similar; the cells became distinctly smaller (Pl. 1, figs. 5, 6). At higher concentrations (*c.* 50–60%) of these alcohols it was observed that the yeast cells formed conspicuous clumps. In each clump the cytoplasm of the cells appeared to consist of a coarse network, which was usually partly retracted from the outer wall.

In the stronger alcoholic media, the cell walls were obviously weakened. This was especially marked with cells in contact with propyl alcohol. The extruded cytoplasm from cells disrupted by mechanical pressure became more heavily stained by nigrosin than the cytoplasm remaining within the cells (Pl. 1, fig. 7).

In the experiments with butyl alcohol, the cells were in contact with a saturated aqueous solution in equilibrium with droplets of excess solvent. After a relatively short period of contact with butyl alcohol a few minutes only, the yeast cells on the slide seemed to shrink slightly, the cytoplasm became more granular and withdrawn locally from the cells walls, and the vacuole disappeared. At a slightly later stage, the cytoplasm became more uniform or homogeneous in appearance, and marked staining by nigrosin was apparent (Pl. 1, fig. 8). At this stage the cells were very easily disrupted by mechanical pressure, resulting in a liberation of cytoplasm, which tended to spread out in the medium from the point of escape through the ruptured cell wall (Pl. 1, fig. 9). This spreading or dispersion of cytoplasm with

mechanical pressure was more easily achieved with cells which had been in contact with butyl alcohol than with those exposed to high concentrations of propyl alcohol and the staining of the cytoplasm remaining within the cells and in intact cells took place more rapidly in the presence of butyl alcohol. In cells which had been in more prolonged contact with butyl alcohol, both the extruded and the internal cytoplasm were heavily stained by nigrosin (Pl. 1, fig. 10.)

While the general action of amyl alcohol was similar to that of butyl alcohol, deformation of the cell, weakening of the cell wall and tendency to dispersion of extruded cytoplasm were less than in the lower homologue, although the degree of cytoplasmic staining in the intact cells was very intense (Pl. 1, fig. 11).

Ethyl alcohol-ether mixture. The shrinkage and distortion of the cells in the mixed solvent system was very pronounced, and in most cells the cytoplasm was partially withdrawn from the cell wall. As observed in cells which had been in contact with chloroform, butyl alcohol or amyl alcohol, the cell wall was greatly weakened after treatment in the mixed solvent system, so that many cells burst when mechanical pressure was applied, and the exposed cytoplasm was quickly stained by nigrosin. The disruptive action of the mixture of solvents was greater than that of either component alone (Pl. 1, fig. 12).

III. *Observations on dried yeast cells before treatment with fat solvents*

In a thin layer of aqueous yeast suspension spread on a slide, the cells were at first uniformly distributed as in a similar preparation where the film was protected from drying by a cover-slip. As evaporation from the watery layer occurred, the cells began to clump together, and the dried cells formed aggregates of characteristic appearance on the surface of the slide. The nigrosin 'shadowing' technique clearly showed the globular shape of the individual cells in the dried clumps (Pl. 2, fig. 1). This appearance corresponded to that of cells viewed from the side after deposition on the rounded edge of a cover-slip (Pl. 2, fig. 2); the cells projected from the glass surface, the curved outer surfaces of the cells being clearly visible.

IV. *Observations on dried yeast cells after treatment with fat solvents*

Nigrosin 'shadowing' technique

Hexane. Cells which had been treated with hexane dried to form clumps of globular cells similar to the dried controls (Pl. 2, fig. 3).

Ether. Here also the cells were generally spherical in appearance, but in some the outer surface of the cell wall showed depressions or corrugations (Pl. 2, fig. 4).

Chloroform. Yeast cells dried after treatment with chloroform did not appear to be spherical after staining with nigrosin, and the appearance of the cells as a whole suggested that during dehydration, a considerable degree of flattening had occurred (Pl. 2, fig. 5).

Ethyl, propyl, butyl and amyl alcohol. After contact with ethyl (Pl. 2, fig. 6) or propyl alcohol (Pl. 2, fig. 7), the yeast cells were somewhat flatter than the controls, this difference being more apparent with propyl than with ethyl alcohol.

The effect of butyl alcohol was much more marked than that of the lower alcohols

(Pl. 2, fig. 8). The cells were considerably flattened, and packed so that their outlines were polyhedra. The cytoplasm was deeply stained with nigrosin, giving the impression that the dye was able to penetrate very readily into the cell interior. The flattened shape of the transparent cells, already referred to, was shown by the absence of any marked shadowing in the peripheral region.

The effect of amyl alcohol (Pl. 2, fig. 9) was similar to that of butyl alcohol, although the cells were circular or oval rather than polyhedral in outline, and they formed smaller clumps. The cytoplasm was fairly heavily stained by nigrosin, and there was no uniform gradation in shadowing, such as is to be seen in the spherical controls, showing that flattening had occurred.

Ethyl alcohol-ether mixture. The dried cells were similar in appearance to those which had been in contact with butyl alcohol (Pl. 2, fig. 10), indicating that the cells were flattened. The staining of the cytoplasm by nigrosin was fairly intense.

Lateral appearance of dried yeast cells

Using the method described in which cells were dried on the rounded edges of cover-slips, direct observations were made of the effects of fat solvents on the degree of cell flattening when the cells were examined from the side. The results agreed closely with those obtained by the nigrosin 'shadowing' method. After treatment with hexane, the cells still retained the globular shape shown by the controls (Pl. 2, fig. 11), and a somewhat similar appearance could be seen in isolated groups of cells which had been treated with ether, although in this case many of the cells did not project so far from the glass surface (Pl. 2, fig. 12).

Pl. 2, fig. 13, shows three cells from a preparation in which the cells have been treated with chloroform. The middle cell is the most flattened; those on either side have flattened to a lesser extent, and their appearance suggests that flattening proceeds from the outside inwards. A number of flattened cells obtained after treatment with butyl alcohol is shown in Pl. 2, fig. 14.

V. *Electron micrographs of cells before treatment with fat solvents*

The object of taking electron micrographs was not the usual object of the electron microscopist, to obtain pictures of cells as nearly as possible resembling the living cell, but on the contrary, as in the experiments with the light microscope already described, to study the gross distortions produced by dehydration, either of unfixed cells or of cells previously treated with various fat solvents.

Although yeast cells were more suitable than *Escherichia* or *Staphylococcus* for examination by light microscopy, they were less suitable for study by electron microscopy owing to their relatively large size (Pl. 3, figs. 1–3). All three types of cells were relatively opaque to the electron beam, and this was mainly due to the presence of the cytoplasm, for where this was withdrawn from the ends of the *Escherichia* cells, the transparent cell walls could be seen in optical section. The degree of retraction varied from cell to cell. Retraction of the cytoplasm was particularly marked in *Staphylococcus* cells (Pl. 3, fig. 1), but the yeast cells were apparently filled with cytoplasm (Pl. 3, fig. 2), and did not show retraction.

VI. *Effects of fat solvents on cell structure shown by electron micrographs*

The clearest indications of the effects of fat solvents on cell structure were obtained with *Escherichia coli*.

Hexane. The general appearance of the cells was unchanged after treatment with hexane, suggesting that if there was any action of the fat solvent on the cell lipids, this was reversible (Pl. 3, fig. 4).

Ether. Cells which had been treated with ether were somewhat wider at the ends than in the middle; the cytoplasm was mainly localized in this region (Pl. 3, fig. 5). In this particular preparation, one cell was oval and completely filled with cytoplasm. In a preparation from a suspension which had been exposed to ether for a longer period (30 min.), the clear zones at the ends of the cells were smaller and the surface of the cytoplasm less sharply defined than in the control cells.

Chloroform. The change which had been noted for the single cell in Pl. 3, fig 5, was general for the cells which had been in contact with chloroform (Pl. 3, fig. 6). The majority of cells were oval and considerably more transparent than the dense parts of the controls; this greater transparency was evident in the central zone of the oval cells.

Ethyl, propyl, butyl and amyl alcohol. The cytoplasm was partially retracted from the cell wall in cells which had been in contact with ethyl alcohol. The cells were rather cone-shaped, with the cytoplasm localized in the apical regions of the cones (Pl. 3, fig. 7). The effect of propyl alcohol (Pl. 3, fig. 8) was similar to that observed for ethyl alcohol. The cytoplasm was withdrawn locally from the ends of the cells; and where this occurred the transparent zones were wider than the parts of the cells filled with cytoplasm. The effects of butyl and amyl alcohol (Pl. 3, figs. 9, 10) were similar to those observed for chloroform. The cells were relatively transparent, filled with cytoplasm, and oval in shape.

Ethyl alcohol-ether mixture. The cells were similar in appearance to those which had been treated with chloroform, butyl or amyl alcohol, although some of the cell walls had ruptured, liberating the cytoplasmic contents.

In all the cells shown in the electron micrographs, the cytoplasm is considerably more opaque than the cell wall, as seen in regions from whence the cytoplasm was retracted. The greater transparency of the cells in Pl. 3, figs. 6, 9 and 10, as compared with that of the control cells in Pl. 3, fig. 3, might be correlated with the flattening of the cells, or with a loss of cell contents or both, in the light of the observations made with the light microscope on the effects of fat solvents on the hydrated and dried cells.

Since both untreated and treated cells were dried before examination, any changes in shape and appearance of those treated with fat solvents can be ascribed to the action of the solvents. These last can be arranged as follows, in descending order according to their ability to increase the transparency and alter the shape of the dehydrated cells: (1) chloroform, ethyl alcohol-ether mixture, butyl alcohol, amyl alcohol; (2) ether, ethyl alcohol, propyl alcohol; (3) hexane. As the homologous series of alcohols is ascended, the relatively pronounced effect of butyl alcohol as

compared with that of propyl alcohol is suggestive of a selective action on cell structure analogous to the lipid-dispersant activity of these alcohols on the lipoprotein structure of the insect epicuticle. In fact, the above solvent order is the same as that for lipid-dispersant activity on the insect epicuticle, as measured by the irreversible increase in permeability and by the increase in the accessibility of the associated phenoloxidase to artificial tanning substrates, such as catechol (Hurst, 1948).

VII. *Electron diffraction diagrams of collodion/lipid membranes and of cells before treatment with fat solvents*

Observations on yeast cells with the light microscope, and on *Escherichia* cells with the electron microscope, had suggested a relationship between the degree of cell flattening and the displacement of lipids from the cells by fat solvents. It was of interest, therefore, to see whether the intensity or nature of the diffraction pattern could be similarly correlated with the degree of cell flattening. The interpretation of the observations made rests on the following structural features of long-chain crystalline substances.

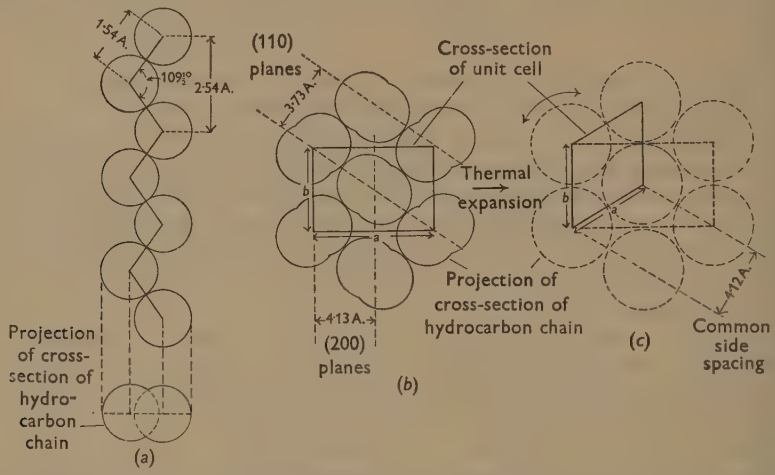
Text-fig. 1*a* shows the zigzag arrangement of carbon atoms in a hydrocarbon chain. The tetrahedral interbond angle is $109\frac{1}{2}°$, the distance between alternate carbon atoms 2·54 A., and the distance between adjacent carbon atoms 1·54 A. (Müller, 1928). In the orthorhombic crystal lattice, where $a=7{\cdot}45$ A., $b=4{\cdot}97$ A. (Müller, 1928), the zigzag chains are arranged in two planes, which facilitates close packing (Text-fig. 1*b*). The theoretical side spacings of the (110) and (200) planes are *c.* 4·13 A. and *c.* 3·73 A., respectively (Brummage, 1947; Hurst, 1950).

The transition from the orthorhombic to the hexagonal form of close packing of the chains is brought about by the anisometric thermal expansion of the unit cell; the lateral expansion along the *a*-axis is greater than that along the *b*-axis. At a critical temperature, though the solid crystalline state persists, the chains are no longer interlocked and become free to rotate; from the crystallographic point of view, the chains behave as freely rotating rods packed in hexagonal symmetry (Müller, 1932). This form of hexagonal close packing is illustrated in Text-fig. 1*c*. The common side-spacing of *c.* 4·12 A. corresponds to a unit cell in hexagonal co-ordinates, where $a=b=4{\cdot}85$ A. (Germer & Storks, 1938). Hurst (1950) has described the transition from the orthorhombic to the metastable hexagonal crystalline structure in long-chain fatty acids (elaidic, brassidic, octacosanoic and tetracontanoic acid), and has shown how the change may be correlated with changes in diffraction transmission pattern of the fatty acids impregnating collodion membranes (see p. 33).

Collodion/yeast-lipid membranes. The diffuse inner ring pattern of the collodion base alone is shown in Pl. 4, fig. 1; it can be seen in all the patterns in Pl. 4, and is quite distinct from the sharp ring patterns of the lipids. The pattern of the fresh collodion/yeast-lipid membrane showed an intense inner ring (*c.* 4·12 A.) and a faint outer ring (*c.* 3·72 A.) (Pl. 4, fig. 2). When the specimen was exposed for 10 min. to a more intense beam (400 μA.) and then examined under the original conditions

(200 μA.), the diffraction pattern was less intense, and consisted of a somewhat diffuse 4·12 A. ring (Pl. 4, fig. 3).

Collodion/phospholipid membranes. The patterns of lecithin (Pl. 4, fig. 4) and kephalin (Pl. 4, fig. 5) were similar to that of the yeast-lipid extract. These patterns also faded when the beam intensity was increased.



Text-fig. 1. Crystalline structure of long-chain hydrocarbon compounds. (*a*) hydrocarbon chain, showing zigzag arrangement of carbon atoms; the projection of the cross-section represents the appearance of the chain from the end-on position; *b*, lateral grouping of hydrocarbon chains in orthorhombic unit cell, seen in cross-section; *c*, lateral grouping of hydrocarbon chains in hexagonal close packing, showing relation to cross-section of unit cell; the freely rotating chains are represented as dotted circles.

The relative intensity of the 4·12 A. ring in the patterns of the collodion/lipid membranes suggests that the hexagonal type of crystalline structure predominated in the membranes. This type of pattern is similar to that of long-chain fatty acids after melting and recrystallization. The weak 3·72 A. ring points to the presence of a small proportion of orthorhombic crystals (Hurst, 1950). Müller (1932), using X-ray diffraction methods, observed the two side-spacings of 4·12 and 3·71 A. in the diffraction pattern of a long-chain crystalline paraffin (C_{24}); the 3·71 A. ring disappeared at the transition point when the orthorhombic lattice changed to one of hexagonal symmetry.

The continuous nature of the rings indicates that the crystals were extremely small, and that the *a*-axes in different crystals were distributed at random in the plane of the membranes. Fading of the patterns with increase in beam intensity was presumably due to the melting of the crystals in which the hydrocarbon chains were originally normal to the plane of the membrane, followed by recrystallization without orientation relative to this plane. This change (thermal disorientation) has been described for long-chain fatty acids (Hurst, 1950), and suggests that the

preferred orientation of the yeast-lipid and phospholipid molecules in the bulk phase of the artificial membrane derives from the orientation of lipid in the adsorbed monolayer at the amyl acetate-water interface.

Yeast and bacterial cells. The diffraction pattern of the control yeast cells (Pl. 4, fig. 6) was similar to that of the control *Staphylococcus* cells (Pl. 4, fig. 10). A faint 4·12 A. ring was present; this could only be seen with difficulty in the pattern of the control *Escherichia* cells (Pl. 4, fig. 14), but its presence was indicated by the sharpness of the outer edge of the inner broad ring as compared with this ring in the pattern of the control collodion base (Pl. 4, fig. 1).

VIII. *Effects of fat solvents on electron diffraction patterns of cells*

Hexane. There was no significant effect of this solvent on the diffraction pattern of yeast (Pl. 4, fig. 17), *Staphylococcus* (Pl. 4, fig. 11), or *Escherichia* cells (Pl. 4, fig. 15), but it must be remembered that a reversible action on the lipids would not be revealed by this technique.

Ether. A slight intensification of the 4·12 A. ring was apparent in the patterns of yeast (Pl. 4, fig. 8) and *Staphylococcus* cells (Pl. 4, fig. 12); the relative degree of intensification was most marked in the pattern of *Escherichia* cells (Pl. 4, fig. 16)

Chloroform. The 4·12 A. ring was much more intense in the patterns of cells which had been treated with ether; an outer faint 3·72 A. ring could also be distinguished. In fact, the patterns of *Staphylococcus* (Pl. 4, fig. 13) and *Escherichia* cells (Pl. 4, fig. 17) were very similar to the patterns of the yeast-lipids and phospholipids (Pl. 4, figs. 2, 4 and 5). This applied also to the pattern of yeast cells (Pl. 4, fig. 9); but here two rectangular grids of spots were present on the 4·12 A. ring, and each grid was associated with a pair of spots on the 4·12 A. ring. The relative dimension of each grid coincided with those of the cross-section of the unit cell in an orthorhombic hydrocarbon chain lattice (Text-fig. 1*b*), showing that there was an alinement of the *a*- and *b*-axes of orthorhombic crystals in two different directions in a plane through the cells normal to the incident beam, each group of alined crystals approximating to a single mosaic crystal (Brummage, 1947; Hurst, 1950).

The patterns did not fade when the cells were exposed to a more intense beam (400 μA.) for 10 min. In this respect the patterns of the cells which had been treated with chloroform differed from those of the collodion/lipid membranes, where fading occurred owing to thermal disorientation.

Ethyl, propyl, butyl and amyl alcohol. These solvents were tested only on *Escherichia* cells. There was a slight intensification of the 4·12 A. ring in the pattern of cells which had been in contact with ethyl alcohol (Pl. 4, fig. 18). The effect of propyl alcohol was similar to that of ethyl alcohol (Pl. 4, fig. 19), but a relatively intense pattern (Pl. 4, fig. 20) was obtained from cells which had been in contact with butyl alcohol, and this was also observed in cells which had been treated with amyl alcohol (Pl. 4, fig. 21). The presence of a faint outer 3·72 A. ring in these patterns showed that they were essentially similar to that of cells which had been initially exposed to chloroform; but there were no rectangular grids of spots on the 4·12 A. ring.

Ethyl alcohol-ether mixture. The combined effect of the two components on diffraction pattern intensification (Pl. 4, fig. 22) was greater than that of the separate components (Pl. 4, figs. 16, 18). The pattern was similar to those of cells which had been treated with chloroform, butyl or amyl alcohol, and in all cases these relatively intense patterns did not fade with increase in beam intensity.

Combining the observations on the different types of cells, it is seen that the fat solvents may be arranged in groups showing the following order of decreasing activity in diffraction pattern intensification: (1) chloroform, ethyl alcohol-ether mixture, butyl alcohol, amyl alcohol; (2) ether, ethyl alcohol, propyl alcohol; (3) hexane. This order coincides with that observed in relation to cell flattening.

DISCUSSION

The value of electron diffraction analysis as a new and sensitive tool in the study of ultra-structure of the cell membrane is illustrated here, where the crystalline structure of the fatty components has been determined in single layers of comparatively few cells. The significance of the experimental evidence will now be considered.

The interpretation of diffraction patterns of polycrystalline lipid membranes

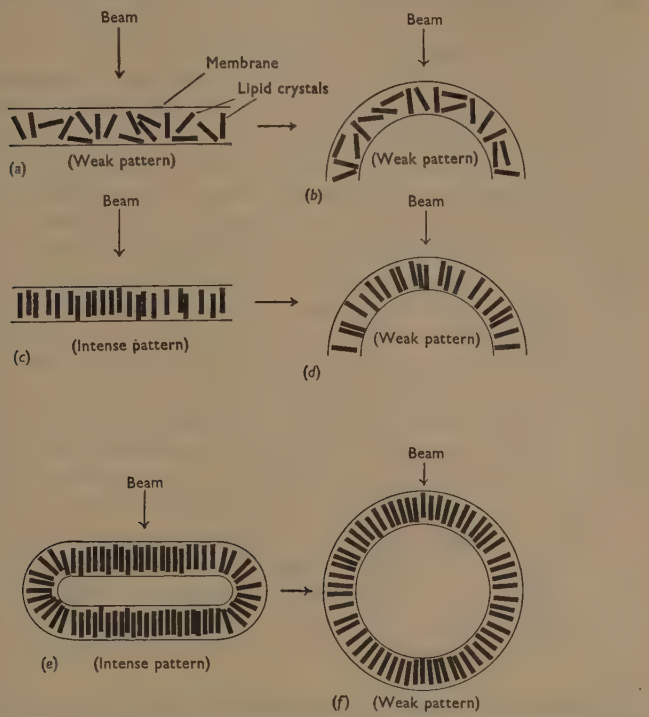
The electron scattering points in crystalline long-chain compounds are the alternate carbon-carbon atoms in the zigzag hydrocarbon chains. The nature of the diffraction pattern depends on the arrangement of the reflecting planes of scattering points in the crystalline lattice. Consider a system in which the electron beam is perpendicular to the plane of a collodion membrane in which small crystals of lipid are distributed at random (Text-fig. 2*a*). Diffraction pattern intensity will depend on the number of crystals which are oriented so that the hydrocarbon chains are almost parallel to the beam. Since the crystals are distributed at random in the area of the membrane covered by the beam, there should be no change in intensity of the rings in the diffraction pattern if the membrane is distorted or bent into a segment of a sphere or cylinder as in Text-fig. 2*b*.

If, on the other hand, the lipid crystals are oriented with the hydrocarbon chains normal to the plane of the membrane and parallel to the beam, the diffraction pattern will be relatively intense, since in all crystals the incident beam will be able to make the correct Bragg angle with all planes of scattering points parallel to itself (Text-fig. 2*c*). Diffraction pattern intensity will decrease if the membrane is distorted or bent into a segment of a sphere or cylinder, since in fewer crystals will the hydrocarbon chains be parallel to the beam. Only in the restricted area of the membrane which is approximately normal to the beam (Text-fig. 2*d*) will the crystals be oriented in this way. In this system, therefore, pattern intensity will vary inversely with curvature of the membrane. Similar relationships between membrane curvature and diffraction pattern intensity will hold for double membranes (Text-fig. 2*e*,*f*).

Cell flattening, diffraction pattern intensification and the action of fat solvents

Any attempt to compare the cell membrane with the hypothetical model systems in Text-fig. 2*e*,*f* is complicated by the fact that the cell membrane consists of the

outer cell wall and the inner cytoplasmic or cell membrane, which is known to contain a relatively high proportion of lipids (Knaysi, 1938). The submicroscopic cytoplasmic particles of yeast cells, isolated by ultra-centrifugation, are also rich in lipids (Nyman & Chargaff, 1949), and since the beam passes through the entire cell,



Text-fig. 2. Collodion/lipid membranes, showing relation between direction of electron beam, shape of membrane, orientation of lipids and intensity of diffraction pattern. *a*, plane of membrane normal to beam; lipids distributed at random in membrane, shown in cross-section; *b*, membrane curved; lipids distributed at random in membrane; *c*, plane of membrane normal to beam; hydrocarbon chains of lipid crystals parallel to beam and normal to plane of membrane; *d*, membrane curved; hydrocarbon chains of lipid crystals normal to surface of membrane; *e*, cross-section of double membrane formed by flattening of spherical membrane; the flattened surfaces are normal to the beam; the hydrocarbon chains of the lipid crystals in these flattened surfaces are parallel to the beam; *f*, spherical membrane, shown in cross-section; the hydrocarbon chains of lipid crystals are normal to the surface of the membrane.

scattering could occur, not only in the outer layers, but also in the cytoplasm itself. The observations on the effects of fat solvents on yeast cells have indicated that flattening is related to the lipid-dispersant action of the solvents on the cell wall. It also seems probable that fat solvents increase the permeability of the cell wall to nigrosin and so facilitate staining of the cytoplasm. (The staining of the cytoplasm in cells which have been in contact with fat solvents will be discussed in detail in

subsequent papers.) If the permeability of the cell wall is increased, cytoplasmic constituents will tend to escape from the cell. An increase in the permeability of the cell wall should therefore cause a decrease in the quantity of lipid in the cell.

The experimental evidence also shows that *Escherichia* cells, which have been treated with ethyl alcohol or ether, flatten locally during dehydration; and that the cytoplasm is withdrawn from the relatively flattened zones into the more cylindrical regions of the cells. The relative increase in intensity of the diffraction pattern after treatment with fat solvents may therefore be related to flattening of the cell wall (p. 38), which is most extreme after treatment with butyl or amyl alcohol, chloroform or a mixture of ether and ethyl alcohol. Since flattening is correlated with an increase in intensity of the electron diffraction pattern, the hydrocarbon chains must be normal to the plane of flattening of the cells. The electron diffraction diagrams thus provide the first direct evidence of what has long been postulated by analogy with the structure of lipid monolayers or micelles, namely, that the lipids of the superficial layers of the cell are oriented with the hydrocarbon chains normal to the plane of the cell surface.

The degree of pattern intensification is proportional to the lipid-dispersant activities of the fat solvents, as measured by their effects on the permeability of the insect epicuticle (Hurst, 1943, 1948). Previous observations on the *Calliphora* pupal skin (Hurst, 1950) have shown that, when the membrane (both before and after treatment in chloroform) is normal to the beam, displacement of fat-soluble lipid results in a weakening of the diffraction pattern, since the proportion of crystalline lipid in which the hydrocarbon chains are parallel to the beam is reduced after displacement of fat-soluble lipid. A similar weakening of the diffraction pattern should occur if the shape of the cells were unchanged by dehydration. This is contrary to experimental observation, the reason being that the cell flattens on dehydration and the proportion of lipid chains oriented parallel to the beam is therefore increased.

The considerable intensity of the diffraction patterns of lipid-extracted cells shows that the proportion of bound lipid in the cell wall is relatively high. Other investigators have indeed reported a high proportion of bound lipid in yeast cells. According to Reichert (1944) more than half the lipids remain when the yeast is treated with fat solvents, but more complete extraction is possible if the yeast is refluxed with 95% methyl alcohol. The bound lipids can be extracted after the lipo-protein structure in which they participate has been broken down by treatment with hydrochloric acid (Grossfeld & Hess, 1943). Rupture of lipo-protein complexes by acid hydrolysis has long been known to increase the proportion of lipids extractable from tubercle bacilli by fat solvents (Long, 1922); and halogenated organic compounds appear to have a similar pronounced dispersant action on the bound lipids of these organisms (Anderson, 1929; Fethke, 1938), which is of interest in view of the marked lipid-dispersant action of chloroform on yeast and bacterial cells described in the present work.

The process of cell-flattening

The shape of yeast and bacterial cells seems to be dependent on the stiffness of the cell wall (Salle, 1943; Bisset, 1950; Clifton, 1950). Weakening of the wall in the presence of fat solvents has been inferred in the present work from the ease with which the treated yeast cells burst under pressure.

Since the stiffness of the cell wall decreases with progressive displacement of lipid, it may be inferred that lipid acts as a stiffening or bonding medium in the cell wall. Moreover, since the lipids are associated with the structural proteins and/or polysaccharides of the wall, a weakening of the lipid structure may be expected to result in a change in the structure of the entire complex of macromolecules constituting the cell wall. Fat solvents may also be expected to interact with non-polar portions of these same macromolecular constituents.

The flattening of the cell during dehydration will be facilitated by a weakening of the cell wall and by the loss of cytoplasmic constituents from the cell. The experimental evidence suggests that both processes occur. Surface tension forces are probably the immediate cause of flattening. When the water film is reduced to the thickness of a single layer of cells, a further decrease in thickness by evaporation will compress the cells between the air-water interface and the supporting solid surface, so that the weaker the cell wall, the greater will be the degree of flattening. In the later stages, the cytoplasm and the cell wall harden owing to dehydration, so that the cell as a whole becomes more rigid and does not revert back to its original shape after the water has evaporated.

This account of flattening is consistent with the observation that aqueous suspensions of yeast and bacterial cells almost invariably dry to form single layers of cells; when cell aggregates form during drying, they are only one cell thick.

For the relatively large yeast cells, the agreement between the appearance of the dried cells as seen by the nigrosin 'shadowing' method, and in side-view when deposited on the edge of a cover-slip, provides unequivocal proof that lipid-dispersant activity of the fat solvents is correlated with the degree of flattening which occurs during dehydration. The electron micrographs of dried *Escherichia* cells suggest that flattening also occurs in these after treatment with fat-solvents, but on account of their small size it would be difficult to confirm this by light microscope observations as for yeast cells. Supporting evidence of extraction of lipids from bacterial cells is provided, however, by the observed changes in the electron transparency of the cytoplasm.

Association between fat solvents and cell-wall lipids

The parallel between the lipid-dispersant activities of fat solvents on the insect epicuticle (a lipo-protein membrane associated with fat-soluble lipid (Hurst, 1943, 1948)), and on the cell wall, points to a similarity in the molecular organization of these different membranes; in particular the effects of the homologous series of primary alcohols are of interest. As the alcohol series is ascended from propyl to butyl, the penetrating and lipid-dispersant power of the alcohol is increased. With

the cuticle of mature blowfly larvae, this effect is seen in the liberation, or increase in accessibility of the cuticle phenoloxidase to an oxidizable substrate such as catechol, which is oxidized to the corresponding *o*-quinone; and the effect is greater with butyl alcohol than with lower or higher homologues (Hurst, 1948).

In the living yeast and bacterial cells, the cell wall is probably intimately associated with the internal cytoplasm; and it is likely that any lipid-dispersant action of fat solvents extends from the cell wall into the more labile cytoplasmic structure. Morton (1950) has noted the parallel between the specific dispersant action of butyl alcohol on the lipo-protein structure of insect epicuticle (Hurst, 1948) and on the lipo-protein structure of cytoplasmic particles associated with a wide range of different enzymes. When the colloidal framework of the insoluble particles is broken down by butyl alcohol, the enzymes are liberated and may be obtained in a more highly purified condition than was hitherto possible.

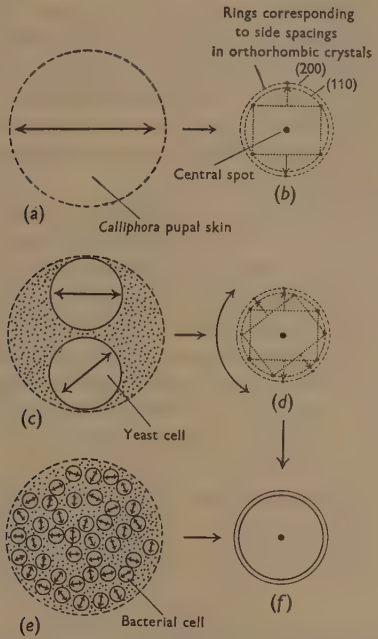
With the lower alcohols (methyl, ethyl and propyl), the van der Waals forces of association between alcohol and lipid are too weak to overcome the lateral adhesion between the hydrocarbon chains of the lipids in the cell wall, so that the disruptive action on the lipids is less pronounced than that of butyl or amyl alcohol. The ether component of the ethyl alcohol-ether mixture may swell the cell-wall lipids and so facilitate the uptake of ethyl alcohol—a joint action typical of that of similar mixtures on insect epicuticle (Hurst, 1940, 1943). The negative effect of hexane on the appearance and on the electron diffraction patterns of yeast and bacterial cells would be accounted for, if its effect on the lipids is reversible. The fact that cells are adsorbed at a hexane-water interface is evidence of association between this solvent and the cell surface.

Crystalline structure of lipids

The dominant feature of the diffraction patterns in Pl. 4 is the 4·12 A. ring which shows that the electron beam covers a random distribution of small hexagonal lipid crystals in which the hydrocarbon chains are parallel to the beam. Such a pattern is given, as we have seen, both by typical long-chain pure fatty acids with a single hydrocarbon chain (Hurst, 1950), and by phospholipids (containing fatty impurities) such as lecithin and kephalin, with two hydrocarbon chains in each molecule. The similarity between the diffraction pattern of extracted yeast lipids, lecithin and kephalin (Pl. 4, figs. 2, 4 and 5) shows that the packing of the molecules is primarily determined by the lateral van der Waals forces between the hydrocarbon chains.

A significant observation arising from the electron diffraction study of cell lipids has been that after treatment with chloroform, butyl alcohol or amyl alcohol, the pattern is essentially identical with that given by the *Calliphora* pupal skin after extraction with chloroform (Hurst, 1950). This pattern consists of an inner ring at *c.* 4·12 A. and an outer, less intense ring, at *c.* 3·72 A. These correspond respectively to the (110) and (200) spacings of the orthorhombic lipid structure, but the greater intensity of the ring at 4·12 A. in the cell pattern indicates that the hexagonal structure predominates in the cell lipids. This observation establishes a similarity between the orientation of the bound lipids in *Calliphora* pupal skin and in

fat-extracted yeast and bacterial cells. In a previous section of the discussion it has been shown that the fat-extracted cells are flattened, and hence a preparation of these consists of a number of double membranes in the plane of the grid at right angles to the beam, and can be compared with a continuous membrane such as the *Calliphora* pupal skin.



Text-fig. 3. Comparison between diffraction patterns of bound lipids in *Calliphora* pupal skin, yeast cell wall and bacterial cell wall. *a*, a portion of a single pupal skin is covered by the beam; the alinement of the *a*- or *b*-axes of the orthorhombic crystals in the plane of the membrane is shown by the arrow; *b*, rectangular grid of spots in pattern of pupal skin; the rectangular grid represents reflexion from the (110) planes; the outer pair of spots represents reflexion from the (200) planes; *c*, two flattened yeast cells are covered by the beam; the alinement of orthorhombic crystals in the plane of each flattened cell is shown by the arrows; *d*, diffraction pattern, consisting of two rectangular grids of spots; each grid corresponds to the alinement of crystals in a single cell; *e*, many flattened bacterial cells covered by the beam; the alinement of the orthorhombic crystals of bound lipid in the plane of each flattened cell is shown by the arrows; *f*, diffraction pattern, consisting of continuous rings, owing to overlapping of spot patterns from separate cells.

The alinement of the *a*- and *b*-axes of the bound lipids in the plane of the *Calliphora* pupal skin is revealed in the diffraction pattern by a rectangular grid of spots on the (110) ring and an associated pair of spots on the (200) ring (Hurst, 1950). Two such groups of spots can be seen in the pattern of yeast cells after treatment with chloroform (Pl. 4, fig. 9), but not in the patterns of *Escherichia* and *Staphylococcus* cells which have been in contact with this solvent (Pl. 4, figs. 13, 17). These differences in pattern may be accounted for in the following way.

The single rectangular grid of spots in the *Calliphora* pattern implies that the oriented bound lipid is distributed in the specimen so that there is an alinement of the *a*- or *b*-axes as in a single mosaic crystal (Brummage, 1947). This alinement is represented by the long arrow in the diagram of the area of pupal skin covered by the beam in Text-fig. 3*a*; the corresponding grid of spots in the diffraction pattern is shown in Text-fig. 3*b*. If there is a similar alinement of crystals in the flattened yeast cell wall, as shown by the arrows in Text-fig. 3*c*, where the beam is supposed to cover two cells, there will be two rectangular grids of spots and associated pairs of spots as in Text-fig. 3*d*. This pattern corresponds to that obtained experimentally (Pl. 4, fig. 9), and it is consistent with the electron micrograph of yeast cells (Pl. 3, fig. 2), which indicates that the beam can cover only two or three cells under the particular experimental conditions.

Since, however, a considerable number of bacterial cells is covered by the beam (Text-fig. 3*e*), even if the crystals were alined as in the yeast cell, the grid patterns of individual cells would overlap to give continuous rings as represented in Text-fig. 3*f*, because the flattened cells are distributed at random in a plane normal to the beam. Such continuous ring patterns are given by *Escherichia* and *Staphylococcus* cells (Pl. 4, figs. 13, 17). The pattern may be derived from that of the yeast cells by rotating the latter pattern about the central spot, as indicated by the curved arrow in Text-fig. 3*d*. If the number of yeast cells in random orientation (as in Text-fig. 3*e*) were increased, the diffraction pattern would be the same as for bacterial cells.

The bound lipids of yeast and bacterial cells, like those of the *Calliphora* pupal skin, are thermo-stable, so that the pattern does not change when the beam intensity is increased. The analogy between the pupal skin and the yeast and bacterial cell walls is even more complete if the fat-soluble lipids of the cells are included; these may be compared with the waterproofing waxes of the insect epicuticle. A comparison between the patterns of control yeast cells (Pl. 4, fig. 6) and those treated with ether (Pl. 4, fig. 8) shows that the 4·12 A. ring is continuous, but sharper and more intense, after a small proportion of lipid has been displaced by ether. Since these patterns are derived from two or three cells only, it is certain that the crystals of fat-soluble lipid in the dried cells are oriented (as are the bound lipids), with the hydrocarbon chains normal to the cell surface, but (unlike the bound lipids) otherwise at random in the plane of this surface. A similar random orientation of waterproofing wax crystals occurs in the *Calliphora* pupal skin, although here the crystals are orthorhombic in structure (Hurst, 1950).

The insect epicuticle and dried yeast and bacterial cell walls appear to have in common a structure consisting of thermo-labile lipids embedded in a thermo-stable lipo-protein framework. It is possible that the lipids in the cell wall are stabilized to some extent by the polysaccharide components present (Dubos, 1949), but the available evidence does not provide any direct indication of the mechanism by means of which the lipids are bound to the supporting membrane framework. Nor is it possible to draw any conclusions as to the precise distribution of the lipids in depth in the cell wall. It is improbable that the bulk of the lipids is present as

a superficial layer, for such an arrangement could hardly contribute to the rigidity of the cell wall; moreover, they would then presumably be easily displaced from the surface by fat solvents.

It must be remembered that what has been demonstrated is a similarity in structure between the insect epicuticle and *dried* cell walls of yeast and bacterial cells. In life the cell walls are hydrated structures, and it is certain, in view of the presence of hydrophilic lipids (particularly the phospholipids), that a proportion of the oriented lipids form hydrated micelles, in which the molecules are highly mobile. Mobile lipid molecules in the micelles will tend to bridge gaps in the bulk lipid phase and so increase its continuity in the cell wall.

This suggestion that the cell wall lipids form a continuous phase is supported by the parallel between the effects of fat solvents in increasing the uptake of nigrosin by yeast cells and in increasing the permeability of the insect epicuticle, where the lipids are known to form a continuous protective layer or layers (Hurst, 1948). It may be inferred from this that a similar continuous layer or layers of lipid is present in the living yeast and bacterial cell wall. That the cell wall itself acts as barrier to nigrosin penetration is shown by the observation that, in the presence of fat solvents, the extruded cytoplasm of yeast cells ruptured by pressure is stained more quickly by nigrosin than the cytoplasm remaining within the cells.

In conclusion it may be pointed out how very remarkable is this analogy, with respect both to lipid structure and permeability, between a membrane bounding the surface of a multicellular organism and membranes of unicellular yeasts and bacteria. The parallel suggests that in spite of obvious differences there exists in the membranes of organisms from widely different groups some common lipo-protein structure, reflected in a common pattern of permeability relationships.

SUMMARY

The structure and orientation of the lipids in the cell walls of yeast, *Staphylococcus aureus* and *Escherichia coli*, has been studied by light microscopy, electron microscopy and electron diffraction methods.

When fat solvents are applied to the hydrated cells in aqueous suspension, the cell walls are weakened owing to the displacement of fat-soluble lipids. If yeast cells are allowed to dry on a glass surface, the degree of cell flattening is proportional to the degree of lipid displacement.

The dried fat-soluble and bound lipids of the cells both show the hexagonal crystalline structure, but a proportion of the bound lipids shows the more condensed orthorhombic structure.

The correlation between the degree of cell flattening and of diffraction pattern intensification shows that there is a preferred orientation of lipid crystals in the superficial layers of the dried cells. The hydrocarbon chains of the lipids are normal to the cell surface, so that a higher proportion of chains is parallel to the electron beam in extracted cells (the cells are flattened in a plane at right angles to the beam), although the total quantity of lipid present has been reduced by treatment with fat

solvent. If flattening did not occur, there should be a weakening of the diffraction pattern, owing to a decrease in the amount of lipid present.

The analogy between the structure of the lipids in the cell wall and in the *Calliphora* pupal skin is discussed.

The electron micrographs and diffraction diagrams were taken with the R.C.A. electron microscope and diffraction unit in the Cavendish Laboratory, Cambridge, by kind permission of Dr V. E. Cosslett. I am particularly indebted to Dr L. E. R. Picken for invaluable advice and criticism; and to Mr R. G. Allen for advice and suggestions during this work. The sample of pure lecithin was kindly supplied by Dr J. H. Schulman, and the kephalin by Dr A. E. Alexander. My thanks are due to Mr G. R. Crowe for technical assistance.

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EXPLANATION OF PLATES

PLATE 1

All photographs on this plate are of yeast cells (*Saccharomyces cerevisiae*) examined in water and in the presence of fat solvents; nigrosin was present in the aqueous suspension medium. They were taken with a Leitz $\frac{1}{12}$th oil-immersion objective and a $\times 10$ eyepiece. Magnification $\times 700$.

Fig. 1. Adsorption of cells at air-water interface. The cells adsorbed at the air-water interface (shown by the heavy black line) are closely packed and compressed. Those in the bulk water phase are less closely packed and are oval in shape.

Fig. 2. Adsorption of cells at hexane-water interface. The selective adsorption of cells at the hexane-water interface is clearly shown by the continuous layer of cells present as compared with the single cell seen in the bulk water phase. Although the cell surface is attracted to the oil surface, the cells are mainly immersed in the water phase, showing that the cell surface is predominantly hydrophilic.

Fig. 3. Cells after contact with ether. The cells are somewhat distorted and irregular in shape. The cytoplasm is conspicuously granular, and the 'sap vacuoles' cannot be seen as in control cells in fig. 1.

Fig. 4. Cells after contact with chloroform. The cells have been subjected to pressure, resulting in the rupture of the cell wall and partial extrusion of cytoplasm. The extruded cytoplasm is more intensely stained by nigrosin than the cytoplasm remaining within the cells. Droplets of chloroform can be seen dispersed in the suspension.

Fig. 5. Cells after contact with ethyl alcohol. The cells have formed a clump in which the individual cells are smaller than those in the controls (fig. 1). The cytoplasm is coarsely granular, and the 'sap vacuoles' have disappeared.

Fig. 6. Cells after contact with propyl alcohol. The cells have formed a clump and are similar in appearance to those which have been in contact with ethyl alcohol (fig. 5).

Fig. 7. Cells after contact with strong (60 %) propyl alcohol. The cells have been subjected to pressure, and the extruded cytoplasm is heavily stained by nigrosin.

Fig. 8. Cells after contact with butyl alcohol. The cells are larger than those which have been in contact with ethyl or propyl alcohol (figs. 5, 6), and the cytoplasm is more homogeneous; staining by nigrosin is also more intense than in intact cells which have been exposed to ethyl or propyl alcohol. The 'sap vacuoles' have disappeared.

Fig. 9. Cells after contact with butyl alcohol. The cells have been disrupted by pressure. The liberated cytoplasm is heavily stained by nigrosin and in some cells has spread out in the medium; the internal cytoplasm is less heavily stained.

Fig. 10. Cells after contact with butyl alcohol. The cells have been disrupted by pressure and photographed at a later stage when both the extruded and internal cytoplasm have become intensely stained by nigrosin.

Fig. 11. Cells after contact with amyl alcohol. The cells have been disrupted by pressure and photographed at a stage when the extruded cytoplasm is more intensely stained by nigrosin than that remaining within the cells.

Fig. 12. Cells after contact with a mixture of ethyl alcohol and ether. The cells have been disrupted by pressure, and both the extruded and internal cytoplasm are rapidly and intensely stained by nigrosin.

PLATE 2

All photographs on this plate are of dried yeast cells (*Saccharomyces cerevisiae*). In figs. 1 and 3–10, the cells are seen dried in the presence of nigrosin. In figs. 2, 11–14, the cells are seen in side-view deposited on the curved edge of a cover-slip. The photographs were taken with a Leitz $\frac{1}{12}$th oil-immersion objective and a × 10 eyepiece. Magnification × 700.

Fig. 1. Control cells, showing globular shape of cells in clump.

Fig. 2. Control cells, showing curved outer surfaces of globular cells projecting from edge of cover-slip.

Fig. 3. Cells after contact with hexane. The cells are globular in shape, as in the controls (fig. 1).

Fig. 4. Cells after contact with ether. The cells are generally spherical in appearance, but in some the outer surface shows depressions or corrugations.

Fig. 5. Cells after treatment with chloroform. The appearance of the cells suggests that a considerable degree of flattening has occurred.

Fig. 6. Cells after contact with ethyl alcohol. The light 'shadowing' of the central zones of the cells suggests that slight flattening has occurred.

Fig. 7. Cells after contact with propyl alcohol. The 'shadowing' of the central zones of the cells is more intense than in cells which have been treated with ethyl alcohol (fig. 6), suggesting that a greater degree of flattening occurs with propyl alcohol.

Fig. 8. Cells after contact with butyl alcohol. The cells are packed so that their outlines are polyhedra. The cytoplasm is very intensely stained by nigrosin, except in some cells where the cytoplasm has escaped. The absence of any marked shadowing in the peripheral region of the cells suggests that a considerable degree of flattening has occurred.

Fig. 9. Cells after contact with amyl alcohol. The cells are oval in outline, and form relatively small clumps. The cytoplasm is fairly heavily stained by nigrosin, and the appearance of the cells as compared with the controls (fig. 1), suggests that flattening has occurred.

Fig. 10. Cells after contact with a mixture of ethyl alcohol and ether. The cells are flattened and similar in appearance to those which have been in contact with butyl alcohol (fig. 8).

Fig. 11. Cells after contact with hexane, showing the globular shape in side-view.

Fig. 12. Cells after contact with ether. For the most part the cells are still globular in shape.

Fig. 13. Cells after contact with chloroform. The preparation shows three cells in side-view. The middle cell is most flattened; those on either side have flattened to a lesser extent.

Fig. 14. Cells after contact with butyl alcohol. The cells are flattened and project only slightly from the glass surface.

PLATE 3

(Electron micrographs)

Fig. 1. Control *Staphylococcus aureus* cells. The opaque cytoplasm in the cells is partially retracted from the relatively transparent cell walls.

Fig. 2. Control *Saccharomyces cerevisiae* cells. The opaque cytoplasm is not retracted from the cell walls.

Fig. 3. Control *Escherichia coli* cells. The opaque cytoplasm is retracted from the ends of some cells.

Fig. 4. *Esch. coli* cells after contact with hexane. The cells are apparently similar to those in the controls (fig. 3).

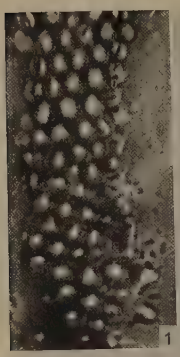
Fig. 5. *Esch. coli* cells after contact with ether. The cells are wider at the ends from which the cytoplasm is withdrawn than in those regions where the cytoplasm is localized. One cell is oval and completely filled with cytoplasm, which is more transparent than that in adjacent cells.

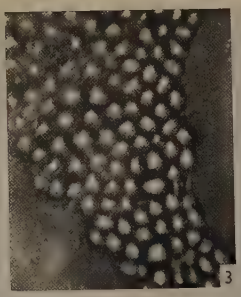
Fig. 6. *Esch. coli* cells after contact with chloroform. The cells are oval and filled with cytoplasm, which is more transparent in the central than in the peripheral zones of the cells; the cytoplasm is generally more transparent than that in the control cells (fig. 3).

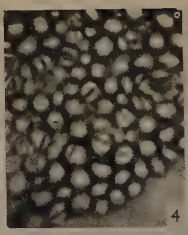
Fig. 7. *Esch. coli* cells after contact with ethyl alcohol. Some of the cells are cone-shaped, with the cytoplasm localized in the apical regions of the cones.

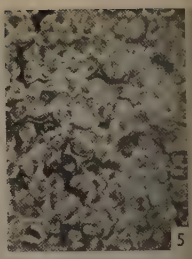
Fig. 8. *Esch. coli* cells after contact with propyl alcohol. The cytoplasm is withdrawn from the ends of the cells.

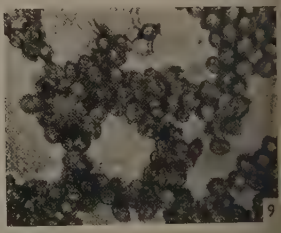
ST—STUDY OF LIPIDS IN YEAST AND BACTERIAL CELL WALLS

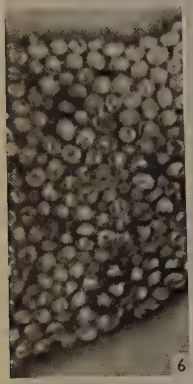


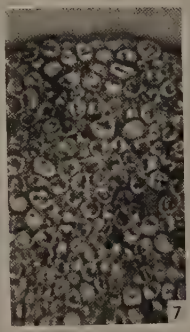


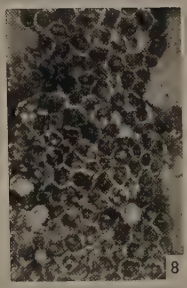


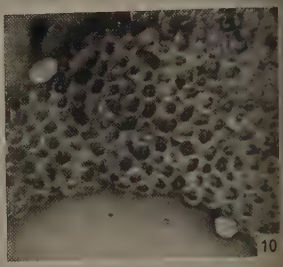


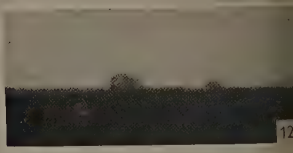


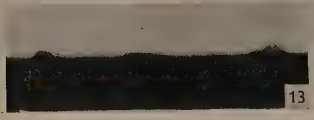


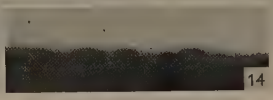




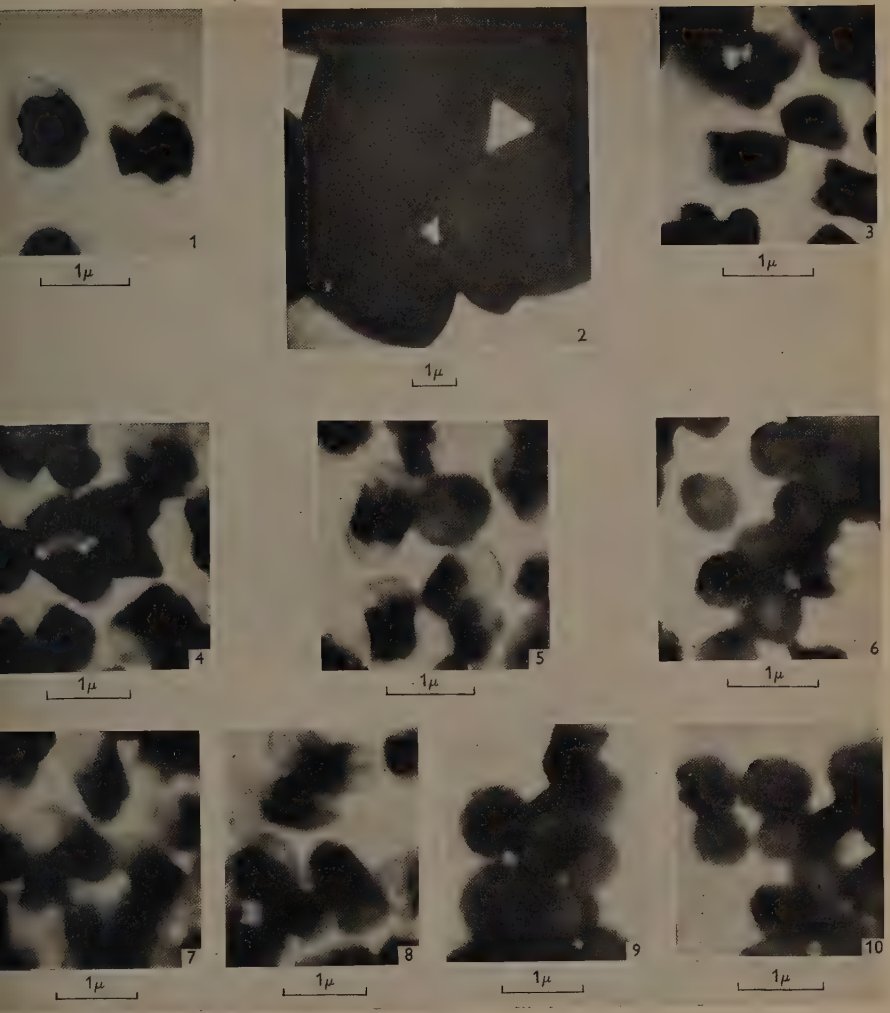








HURST—STUDY OF LIPIDS IN YEAST AND BACTERIAL CELL WALLS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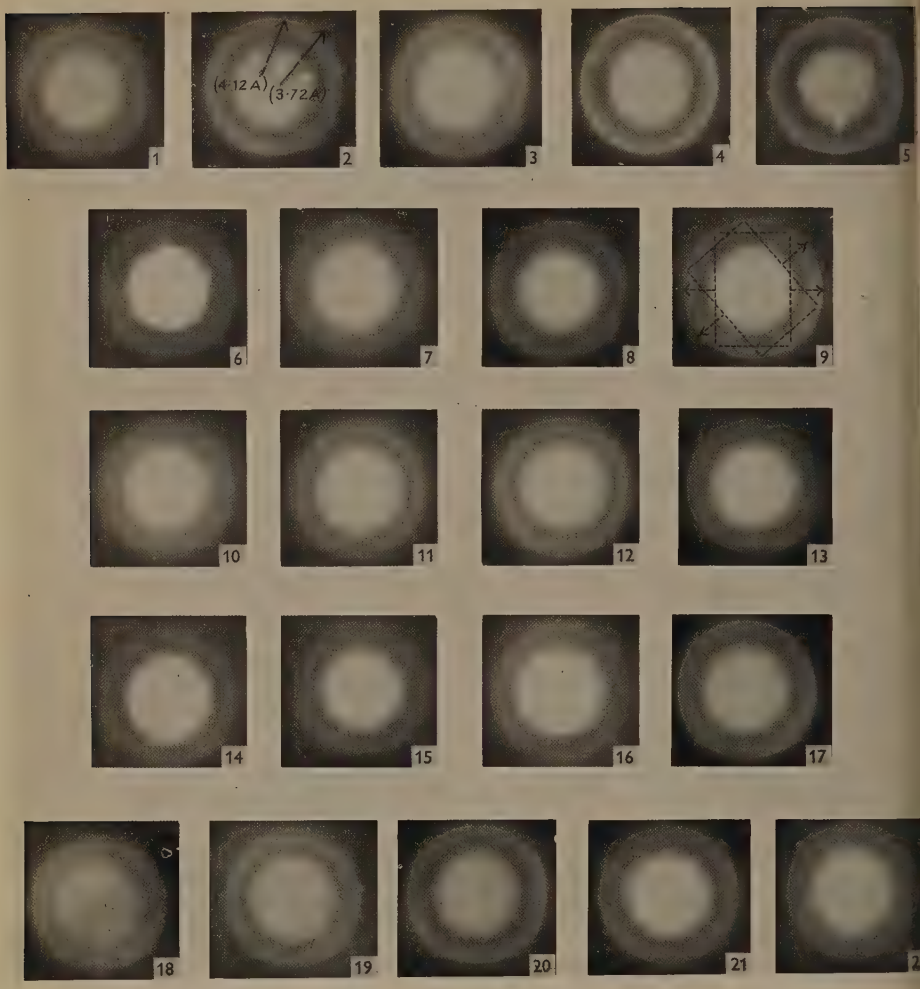

HURST—STUDY OF LIPIDS IN YEAST AND BACTERIAL CELL WALLS

Fig. 9. *Esch. coli* cells after contact with butyl alcohol. The oval cells are filled with cytoplasm, and are similar in appearance to those which have been in contact with chloroform (fig. 6).

Fig. 10. *Esch. coli* cells after contact with amyl alcohol. The oval cells are similar in appearance to those which have been treated with chloroform (fig. 6) or butyl alcohol (fig. 9).

PLATE 4

(Electron diffraction patterns. A weak 3·72 A. ring can be seen in the original patterns in figs. 2, 4, 5, 9, 13, 17 and 20–22.)

Fig. 1. Control collodion membrane. There is a diffuse ring outside the central spot.

Fig. 2. Collodion yeast-lipid membrane. There is a sharp continuous 4·12 A. ring and a faint outer 3·72 A. ring. The rings are indicated by the arrows.

Fig. 3. Collodion-yeast lipid membrane, after thermal disorientation of lipid components. Only the 4·12 A. ring is present, but it is more diffuse than that in fig. 2.

Fig. 4. Collodion lecithin membrane. The pattern is similar to that of the collodion-yeast lipid membrane (fig. 2).

Fig. 5. Collodion kephalin membrane. The pattern is similar to those in figs. 2, 4.

Fig. 6. Control *Saccharomyces cerevisiae* cells. A rather weak 4·12 A. ring is present.

Fig. 7. *S. cerevisiae* cells after contact with hexane. The pattern is similar to that of the control cells (fig. 6).

Fig. 8. *S. cerevisiae* cells after contact with ether. The 4·12 A. ring is sharper than that in fig. 6.

Fig. 9. *S. cerevisiae* cells after contact with chloroform. The 4·12 A. is intensified and shows two rectangular grids of spots. A faint outer 3·72 A. ring is also present; each pair of spots on this ring is associated with a pair of spots on the inner ring. This arrangement is indicated by the dotted lines.

Fig. 10. Control *Staphylococcus aureus* cells. A weak 4·12 A. ring is present.

Fig. 11. *Staph. aureus* cells after contact with hexane. The pattern is similar to that of the control cells (fig. 10).

Fig. 12. *Staph. aureus* cells after contact with ether. The pattern is similar to, but more intense than, that of the control cells (fig. 10).

Fig. 13. *Staph. aureus* cells after contact with chloroform. The 4·12 A. ring is relatively intense, and is surrounded by a faint 3·72 A. ring.

Fig. 14. Control *Escherichia coli* cells. An extremely weak 4·12 A. ring is present, which makes the outer edge of the diffuse ring of the collodion pattern sharper than that in the pattern of the control membrane (fig. 1).

Fig. 15. *Esch. coli* cells after contact with hexane. A weak 4·12 A. ring is present.

Fig. 16. *Esch. coli* cells after contact with ether. The 4·12 A. ring is more intense than that in fig. 15.

Fig. 17. *Esch. coli* cells after contact with chloroform. The 4·12 A. ring is very intense, and is surrounded by a weak, but sharp 3·72 A. ring.

Fig. 18. *Esch. coli* cells after contact with ethyl alcohol. There is a weak 4·12 A. ring.

Fig. 19. *Esch. coli* cells after contact with propyl alcohol. The 4·12 A. ring is stronger than that in fig. 18.

Fig. 20. *Esch. coli* cells after contact with butyl alcohol. The 4·12 A. ring is intense, and is surrounded by a weak 3·72 A. ring as in fig. 17.

Fig. 21. *Esch. coli* cells after contact with amyl alcohol. The pattern is similar to that in fig. 20.

Fig. 22. *Esch. coli* cells after contact with a mixture of ethyl alcohol and ether. The intense pattern is similar to those in figs. 13, 17, 20, 21.

FERMENTATION IN THE RUMEN OF THE SHEEP

III. INTERMEDIATE STAGES IN THE FERMENTATION OF WHEATEN HAY *IN VITRO* BY MICRO-ORGANISMS FROM THE RUMEN

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(With One Text-figure)

A previous communication in this series of papers (Gray, Pilgrim & Weller, 1951) gave an account of the fermentation of wheaten hay and lucerne hay by organisms from the rumen of the sheep, in which the overall production of each of the main volatile fatty acids was determined. In view of the complex nature of both the substrate and the mixed population of organisms involved, it was decided to investigate the intermediate stages of the fermentation to determine whether any important changes take place in the composition or in the rate of production of the mixture of acids.

EXPERIMENTAL

Groups of eight fermentations were set up in flasks, each of which contained the same quantity of a uniform sample of wheaten hay; the fermentations were initiated by the addition of equal volumes of rumen fluid.

Fermentation flasks. 250 ml. Erlenmeyer flasks fitted with inlet tubes reaching nearly to the bottoms of the flasks and outlet tubes arranged for the escape of gas.

Substrate. 5 g. ground wheaten hay chaff.

Inoculum. 1500 ml. rumen fluid were drawn $1\frac{1}{2}$ hr. after feeding the sheep on wheaten hay chaff. To this was added a solution of 6 g. $(NH_4)_2CO_3$ in 200 ml. of tap water. Each flask was inoculated with 150 ml. of the resulting liquor.

Anaerobiosis. Immediately after inoculation a stream of CO_2 was passed through each flask to displace air, and the flasks were sealed by means of a water trap.

Incubation. 38–40° C.

Samples for analysis of volatile fatty acids. 100 ml. of the inoculum were retained for analysis. At intervals during the period from 0 to 48 hr. after inoculation a flask was taken from the incubator, the solids removed by filtration, and the remaining liquor used for the analysis.

Analyses. The methods for the separation and determination of the individual fatty acids have been described previously (Gray *et al.* 1951).

Results. The analytical data from one typical experiment are summarized in Table 1. The composition of the acid mixture at various stages, after allowing for

Table 1. *Fatty acids present in the inoculum and in the fermentation flasks*

	Time (hr.)	ml. N/100 acid/ml. of fluid			
		Acetic	Propionic	Butyric	Total
Inoculum	0	3·02	1·10	0·65	4·77
Flask 1	2	3·33	1·47	0·69	5·49
Flask 2	4½	4·21	2·44	0·91	7·56
Flask 3	7½	5·77	3·28	1·13	10·18
Flask 4	12½	6·43	3·68	1·39	11·50
Flask 5	19½	6·68	3·83	1·38	11·89
Flask 6	28½	7·21	4·20	1·54	12·95
Flask 7	37½	7·80	4·54	1·74	14·08
Flask 8	48	8·50	4·77	1·96	15·23

the acids added with the inoculum, is given in Table 2. The changes in composition of the acid mixture, and the progressive total amounts of acid formed, are illustrated graphically in Fig. 1.

DISCUSSION

In the first place it can be seen that the overall production of fatty acids, as illustrated by the 48 hr. sample, followed a course similar to that taken by the fermentations on a larger scale previously described (Gray *et al.* 1951). Secondly, although the composition of the fatty acid produced showed a change from a higher to a lower proportion of propionic acid during the period of maximum rate of production, it is important to note that in all stages of the fermentation the composition differed from the composition of the mixture present in the rumen fluid in that it contained a significantly greater proportion of propionic acid. Thirdly, it is clear that the rate of production of fatty acid was rapid at first (60% of the total acid was formed in the first quarter of the fermentation period) and thereafter declined to a lower but fairly steady level.

Table 2. *Fatty acids formed during various periods of the fermentation*

Period (hr.)	ml. N/100 acid/ml. of fluid				Percentages of individual acids		
	Acetic	Propionic	Butyric	Total	Acetic	Propionic	Butyric
0–2	0·31	0·37	0·04	0·72	43	51	6
0–4½	1·19	1·34	0·26	2·79	43	48	9
0–7½	2·75	2·18	0·48	5·41	51	40	9
0–12½	3·41	2·58	0·74	6·73	51	38	11
0–19½	3·66	2·73	0·73	7·12	52	38	10
0–28½	4·19	3·10	0·89	8·18	51	38	11
0–37½	4·78	3·44	1·09	9·31	51	37	12
0–48	5·48	3·67	1·31	10·46	52	35	13

SUMMARY

In an investigation of the intermediate stages of the rumen fermentation of wheaten hay *in vitro*, the proportion of propionic acid in the fatty acid products was shown to decrease during the first few hours, when the rate of production of acid was at

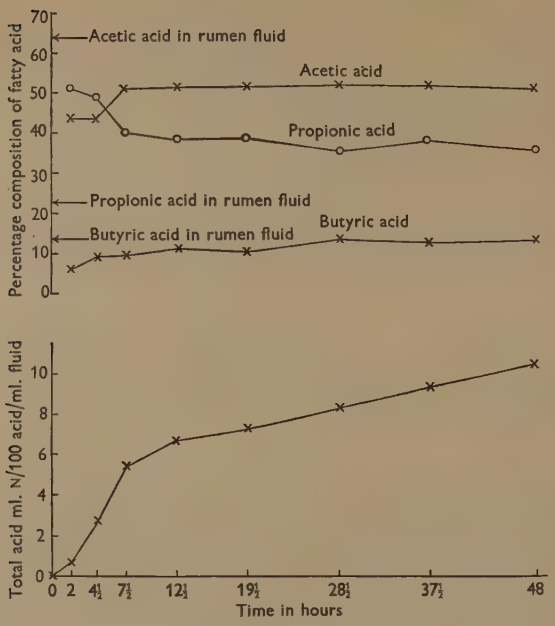

Fig. 1. Composition and total amount of the fatty acids formed at various stages of the fermentation *in vitro*.

a maximum. The composition and the rate of production were uniform over the rest of the fermentation period. Throughout the fermentation the proportion of propionic acid was considerably higher than in the rumen fluid.

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FERMENTATION IN THE RUMEN OF THE SHEEP

IV. THE NATURE AND ORIGIN OF THE VOLATILE FATTY ACIDS IN THE RUMEN OF THE SHEEP

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Early workers in the field of ruminant physiology were aware of the presence of formic, acetic, propionic and butyric acids (Tappeiner, 1884), and of smaller amounts of higher members of the saturated fatty acid series (Mangold, 1934), in the rumen fluid. Accurate data were not reported, however, until recently, when partition chromatography became available as an analytical procedure for the analysis of complex mixtures of these acids (Elsden, 1946). By this means acetic, propionic and butyric acids were established as the main components of the mixture of fatty acids in the rumen fluid of sheep and of other ruminants. The butyric acid fraction, however, was thought to contain some higher acid or acids, and it was claimed that formic acid was not a component of the mixture (Elsden, 1945). Using modifications of the older partition and distillation methods Gray (1946) found similar proportions of the three main acids, but also stated that small amounts of formic acid were normally present.

Earlier communications in this series (Gray, Pilgrim & Weller, 1951; Gray & Pilgrim, 1951) dealt with the production of acetic, propionic and butyric acids during the rumen fermentation *in vitro* and *in vivo*. A more detailed analysis of the fatty acids of the rumen fluid is reported in the present work, and an attempt has been made to trace the origins of some of these acids by incorporating ^{14}C-labelled acetic and propionic acids into the inoculum used to initiate the fermentation of wheaten hay and lucerne hay *in vitro*. The investigation was prompted by the observation that when such labelled acids were introduced into the normal rumen, some of the labelled carbon appeared later in the 'butyric' fraction of the fatty acid mixture.

EXPERIMENTAL

(1) The procedures used in the collection of rumen fluid and of fermentation liquor, and the method employed for separation of the fatty acids from other substances have been described in an earlier publication (Gray *et al.* 1951).

(2) *Methods for the separation, identification and determination of the saturated fatty acids* C_1 *to* C_7. The acids were separated from one another by partition chromatography, using three different columns:

Column 1. For separation into (*a*) formic, (*b*) acetic, (*c*) propionic and (*d*) the

remaining higher acids. Preparation and use of the column have already been described (Gray *et al.* 1951).

Column 2. For separation of the group of higher acids from the first column into (*a*) butyric, (*b*) valeric and (*c*) higher acids. The column was prepared in two parts, separation being effected in the upper, buffered portion (cf. Moyle, Baldwin & Scarisbrick, 1948). *The lower portion of the column* was similar to column 1, but the indicator used was Brom-cresol purple. Immediately before use, dilute ammonia was added to a solution of 0·2 mg./ml. of this indicator, in just sufficient quantity to give a purple colour; 1·5 ml. of the resulting solution was added to 2·5 g. of 'Celite' in a mortar and mixed thoroughly and rapidly. Developing solvent (BB7·5, as used in column 1) was added to form a slurry before sufficient atmospheric CO_2 had been absorbed to change the colour from purple to yellow. The mixture was packed in the column (12 mm. diameter) to a height of 5 cm. *The upper portion of the column* was made by mixing 2 ml. of 2M-K_2HPO_4 and 1 ml. of 2M-KH_2PO_4 with 5 g. celite. A slurry was prepared with BB7·5 and the column packed to a height of 10 cm. above the indicator portion. The column was made ready for use by passing through it a mixture of acids of approximately the same composition as the mixture to be examined. Butyric, valeric and the group of higher acids became visible as yellow bands passing through the lower portion of the column. The eluates were titrated with N/100-barium or sodium hydroxide in a CO_2-free atmosphere, using phenol red as indicator.

Column 3. For separation of the higher acids eluted from column 2, into (*a*) caproic and (*b*) heptoic and higher acids. This column differed from the previous one only in the reaction of the buffered portion, which contained 2M-K_2HPO_4 alone.

Preparation of acids for columns 2 *and* 3. The group of acids eluted from column 1 was neutralized (phenol red) with sodium or barium hydroxide and taken to dryness on a steam-bath in a 10 ml. centrifuge tube. Ten drops of wet BB7·5 were added, and then sufficient dry powdered potassium bisulphate was stirred in to produce a pink colour in the mixture. After centrifuging for a short time, a small bulb-pipette was used to transfer the liquid to the column. The extraction was repeated about ten times, using sufficient BB7·5 to give a total volume of extract of about 1 ml.

Behaviour of pure acids on columns 2 *and* 3. Approximate determinations of *R* values were made by measuring the total movement of the surface of the developer required for the leading edges of the bands to reach the centre of the indicator portion of the column. As these *R* values varied considerably with acid concentration, the relative amounts of pure acids used were of the same order as those found in rumen fluid.

Column 2. *R* determinations on pure acids and mixtures showed that the first rapidly eluted band contained caproic and higher acids. The *n*-valeric band appeared next, followed by the other valeric isomers. These isomers could not be clearly separated unless concentration relationships were ideal. The butyric acids appeared well behind the valeric band, but as the *R* values of *iso*- and *n*-butyric acids were almost identical, no separation could be obtained. Propionic acid was firmly held in the buffered portion of the column.

Column 3. Amounts greater than 0·6 mg. of heptoic acid were necessary to produce a visible band on the column. This band was eluted well ahead of the caproic band and excellent separations were possible. Valeric acid moved very slowly through the buffered column and was not easily eluted.

Recovery of acids. Some acid is not recovered in the first use of these celite columns, and they should therefore be prepared for use by passing through them a mixture of acids similar in composition to the mixture to be separated. Even with this precaution recoveries varied from 86 to 96% on column 2 and from 67 to 100% on column 3. They were, however, very nearly complete on column 1.

Identification and determination of iso-*butyric acid.* The presence of *iso*-butyric acid in the mixture of the two butyric acid isomers was demonstrated by applying the test described by Ramsey & Patterson (1945) in which *iso*-butyric acid is oxidized to acetone by acid permanganate in the presence of silver sulphate. The acetone, after distillation, gives a red colour with salicylaldehyde. This procedure, when carried out under strictly standardized conditions, provided a quantitative colorimetric determination of *iso*-butyric acid. Pure *n*-butyric and *iso*-butyric acids were used to prepare a series of standards in which the varying quantity of *iso*-butyric acid amounted to 5% of the total butyric acid in each case. The standards, together with a blank and the salt under test were oxidized and distilled, and the distillates used simultaneously to develop the colours with salicylaldehyde. After cooling, the colour densities were measured in a spectrophotometer (wave-length 530 mμ) and the amount of *iso*-butyric acid in the salt determined graphically. The presence of barium did not affect the acetone yield.

Formic acid in the lowest boiling fraction of the mixture of acids present in the steam distillate from rumen fluid was identified by its conversion to formaldehyde and the colour reaction with chromotropic acid (Feigl, 1939). Estimation by this procedure (Grant, 1948) and by a gravimetric method involving reduction of mercuric chloride (Scott, 1925) gave similar results.

(3) *Incorporation of* ^{14}C-*labelled acetic and propionic acids in the rumen fermentation* in vitro. The procedure for reproducing the rumen fermentation of wheaten hay and lucerne hay, *in vitro*, has already been described (Gray *et al.* 1951). In the present experiments, 25 g. of the fodder were used and approximately 750 ml. of rumen fluid were employed as inoculum. To this inoculum was added a small amount of either acetic or propionic acid labelled with ^{14}C in the carboxyl carbon. The active acids were prepared by carbonation of the Grignard reagent in the usual manner. In the majority of the experiments enough of the labelled acid was added to give an activity of approximately 200 counts/min./ml. of inoculum, after plating out in the manner described below; in two of the experiments a much larger amount of the active acid was added. Six of the fermentations were made with wheaten hay as substrate and one with lucerne hay.

(4) *Measurement of the activities of the acids.* The neutral solutions obtained by titration of the acid fractions from the chromatographic columns were brought to dryness on a water-bath. The residues were taken up in exactly 2 ml. of water and aliquots (0·5 ml.) of these solutions were spread uniformly on plates of oxidized

Table 1. *Fermentations in the presence of* $CH_3{}^{14}COOH$ *or* $CH_3CH_2{}^{14}COOH$. *The fatty acids and their activities in the rumen fluid used as inoculum, and in the fermentation liquor remaining at the end of the fermentation* in vitro

Exp. no.	Fodder fermented*	Fluid analysed	Acids present (ml. N/100 volatile acid/ml. of fluid) Activities of acids (counts/min./ml. of fluid)†									
			Acetic acid		Propionic acid		Butyric acid		Valeric acid		Caproic and higher acids	
1	Wheaten hay, sample 1	Inoculum	4·30	**242**	1·37	—	←		0·77	—	→	
		Fermentation liquor	9·00	**152**	4·68	**18**			3·13	**78**		
2	Wheaten hay, sample 1	Inoculum	3·53	**196**	1·28	—	0·50	—	0·12	—	0·02	—
		Fermentation liquor	7·72	**114**	4·46	**17**	1·56	**33**	0·70	**10**	0·17	**3**
3	Wheaten hay, sample 1	Inoculum	3·84	**194**	1·31	—	0·52	—	0·14	—	0·06	—
		Fermentation liquor	8·18	**128**	4·41	**13**	1·62	**32**	0·75	**14**	0·31	**6**
4	Wheaten hay, sample 2	Inoculum	3·60	**1790**	0·83	—	0·57	—	0·12	—	0·05	—
		Fermentation liquor	8·13	**1306**	3·50	**90**	1·28	**169**	0·34	**25**	0·20	**15**
5	Lucerne hay	Inoculum	5·00	**207**	1·68	—	0·50	—	0·24	—	0·05	—
		Fermentation liquor	10·28	**175**	4·74	**10**	1·13	**20**	0·69	**6**	0·23	**2**
6	Wheaten hay, sample 1	Inoculum	3·50	—	1·51	**200**	0·48	—	0·09	—	0·05	—
		Fermentation liquor	7·77	Nil	4·82	**149**	1·59	Nil	0·48	**13**	0·25	**3**
7	Wheaten hay, sample 2	Inoculum	4·40	—	1·52	**1710**	0·57	—	0·15	—	0·05	—
		Fermentation liquor	7·83	**20**	4·09	**1246**	1·59	Nil	0·50	**95**	0·22	**8**

* The same type of fodder, but not the same sample, was fed to the sheep from which the inocula were taken.
† Given in black figures.

copper 2·3 cm. in diameter with a central depression of 1·9 cm. diameter. The contents of the plates were evaporated to dryness on a hot plate (65–70° C.) and finally heated in an air oven at 120° C. for 15 min., and the prepared plates were kept in a desiccator over $CaCl_2$ until counted. Preliminary trials indicated that reproducible films could be obtained in this way. Counting was carried out using a shielded end-window G-M tube (background 8 counts/min.) which was standardized during each series of counts against a standard of uranium glass. Corrections for thickness of sample were made in the usual manner from an absorption curve previously prepared. The corrected activities reported are the means of triplicate samples.

(5) *Analyses.* In Exp. 1 the acids were separated into three groups only—acetic, propionic, and higher acids. In Exps. 2, 3, 5 and 6 the butyric and valeric acids were separated but the caproic and higher acids were measured as a group, while in Exps. 4 and 7 the final separation of caproic from higher acids was also carried out. In Exps. 3, 5 and 6, the butyric fraction was recovered after its activity had been measured and the proportions of *iso*-butyric and *n*-butyric acids were determined. In Exp. 5 two isomers of valeric acid were separated, one of which was *n*-valeric acid. Formic acid was not determined in any of the *in vitro* experiments.

(6) *Results.* The amounts and activities of the fatty acids in the inocula and in the liquors remaining at the end of the fermentation period are summarized in Table 1. The molecular proportions of the individual acids in the rumen fluid used as inocula in these experiments are given in Table 2. This table also includes the further analyses of the butyric, valeric and caproic fractions.

Table 2. *Molecular proportions of fatty acids in the rumen fluid 1–2 hr. after feeding*

Exp. no.	Fodder	Acetic (%)	Propionic (%)	Butyric (%)	Valeric (%)	Caproic and higher acids (%)	Further separations
1	Wheaten hay	66·8	21·3	←	11·9	→	—
2	Wheaten hay	64·7	23·5	9·2	2·2	0·4	—
3	Wheaten hay	65·4	22·3	8·9	2·4	1·0	*n*-Butyric, 8·6 %; *iso*-butyric, 0·3 %
4	Wheaten hay	69·6	16·0	11·0	2·4	1·0	'Heptoic', 0·04 %*
5	Lucerne hay	66·9	22·5	6·7	3·2	0·7	*n*-Butyric, 6·1 %; *iso*-butyric, 0·6 %; *n*-valeric, 2·1 %
6	Wheaten hay	62·2	26·8	8·5	1·6	0·9	*n*-Butyric, 8·1 %; *iso*-butyric, 0·4 %
7	Wheaten hay	65·9	22·7	8·5	2·2	0·7	'Heptoic', 0·05 %*

* Approximate values.

DISCUSSION

(1) *The nature of the fatty acids.* The experiments have established the presence of formic, acetic, propionic, *n*-butyric, *iso*-butyric, valeric, caproic and probably heptoic acid in the rumen fluid of sheep fed on wheaten hay or on lucerne hay.

In the case of lucerne hay the valeric acid was present as *n*-valeric and another isomer, the identity of which was not finally established.

(2) *The proportion of each acid in the rumen fluid.* The proportions in which the various acids are present in the rumen fluid are shown in Table 2. It must be stressed that these proportions were found in fluid drawn soon after feeding, for it has already been reported (Gray & Pilgrim, 1951) that the composition of the mixture changes subsequent to feeding, the proportion of propionic acid increases and that of acetic or butyric acid decreases for some hours, after which these changes are reversed and the mixture gradually returns to its original composition. The presence of formic acid was again established, but it was not determined quantitatively in the *in vitro* experiments. However, numerous analyses of rumen fluid (Gray & Pilgrim, 1947–8) have shown that it may constitute 0–5% of the total fatty acid present. In three of the experiments, the *iso*-butyric acid was determined and found to vary from 3 to 8% of the total butyric acid production. In the experiment in which lucerne hay was fermented, the valeric acid contained a fraction identified by its *R* value to be *n*-valeric, the quantity of which accounted for over 60% of the total valeric acid present.

In Exps. 4 and 7 the caproic acid was separated from a faster moving acid which may have been heptoic acid. The amount of this last acid was quite small, sufficient to titrate but showing as a definite band only in Exp. 7. It was present to the extent of about 10% of the caproic acid. The composition of the volatile acids of the rumen fluid* may be summarized as follows:

	%		%
Formic acid	0– 5	*iso*-Butyric acid	0·3 –0·6
Acetic acid	62–70	Valeric acid	1·6 –3·2
Propionic acid	16–27	Caproic acid	0·5 –1·0
n-Butyric acid	6–11	'Heptoic' acid	0·04–0·05

(3) *The origin of the fatty acids.* The intermediate steps leading to the formation of volatile fatty acids in the rumen have as yet received little consideration. Many organisms have been subcultured from rumen material and their attack on cellulose *in vitro* has been examined, with varying results, but in view of our more recent knowledge of the organisms responsible for cellulose digestion in the rumen most of this work may be disregarded (cf. Sypesteyn, 1948). However, the production of acetic and propionic acids from pure cellulose by rumen organisms *in vitro* has been demonstrated (Elsden, 1945; Marston, 1948), and two organisms isolated from the rumen, which have strong claims to be active cellulose fermenters there, have

* It is interesting that the feeding of two very different fodders such as lucerne hay and wheaten hay should lead to much the same mixture of acids in the rumen, for there is strong evidence that the proportions of various acids actually produced are by no means the same with these two substrates (Gray *et al.* 1951). The same phenomenon has been noticed by other workers (Elsden, Hitchcock, Marshall & Phillipson, 1946; McClymont, 1951) for fodders of even wider difference; moreover, it has been shown that the same general composition applies for different animals and for different parts of the alimentary tract where plant structures are digested. It is a matter for speculation whether this mixture represents an equilibrium brought about by the factors which govern the relative rates of absorption of the acids through the wall of the gut. There is a clear—but maybe quite fortuitous—parallel between the proportions of the acids in the mixture and their distribution coefficients between oil and water.

been studied in detail (Sypesteyn, 1948). One of these organisms was shown to produce from cellulose a mixture of propionic and acetic acids, and the other a mixture of succinic and acetic acids. Later it was demonstrated (Johns, 1949; Elsden & Sypesteyn, 1950) that succinic acid is readily decarboxylated to propionic acid either by suspensions of rumen organisms, or when introduced into the rumen itself.

Table 3. *Relative activities of the acids in the fermentation*

Exp. no.*	Fodder	Acetic	Propionic	Butyric	Valeric
2	Wheaten hay	100*	15	89	49
3	Wheaten hay	100*	13	88	69
4	Wheaten hay	100*	10	72	34
5	Lucerne hay	100*	11	110	46
6	Wheaten hay	Nil	100*	Nil	41
7	Wheaten hay	0·8	100*	Nil	38

*The mean molar activities of the originally labelled acids have been given the value of 100 and the molar activities of the other acids formed in the fermentation have been calculated accordingly.

It is notable that there is no evidence for the production of significant amounts of fatty acids higher than propionic, from cellulose. The present experiments suggest that a very considerable part of the higher fatty acids in the rumen fluid may be produced by secondary reactions from both acetic and propionic acids. It will be seen from the data in Table 1 that at the end of the fermentation some of the carbon originally present in the carboxyl group of acetic acid is distributed among all of the higher fatty acids that were examined, while part of the carboxyl carbon of propionic acid appears in valeric but not in butyric acid. The extent to which this occurs is best shown by comparing the specific (i.e. molar) activities of the acids formed during the fermentation with the mean molar activity of the acid originally labelled with ^{14}C. The comparison is made in Table 3 where the mean molar activity of the acetic acid in Exps. 2–5 and of the propionic acid in Exps. 6 and 7 is given the value of 100 in each case. It will be seen from this table that when labelled acetic acid was added to the inoculum the molar activity of the butyric acid produced in the fermentation averaged 90%, and the valeric acid 49%, of the mean activity of the acetic acid. When labelled propionic acid was included in the inoculum, however, the butyric acid formed contained no active carbon at all, while the valeric acid was active to the extent of 40% of the mean molar activity of the propionic acid.

These findings suggest very strongly that a large part of the butyric and valeric acids may be formed from the lower acids by the mechanism which has recently been studied in considerable detail in the synthesis of higher volatile fatty acids by *Clostridium kluyveri* (Bornstein & Barker, 1948; Stadtmann, Stadtmann & Barker, 1949); further work would be necessary, however, to establish this as a fact.

It should be noted, incidentally, that some of the carboxyl carbon of acetic acid regularly appeared in the propionic acid formed, and that practically all (90–100%) of the labelled carbon in acetic acid was recovered in the various fatty acids at the

end of the fermentation. Rather less (64–83 %) of the ^{14}C in propionic acid was recovered.

The presence of small amounts of isomers of *n*-butyric and *n*-valeric acid is interesting for it is known that they occur as products of the amino-acid metabolism of a number of anaerobes of the *Clostridium* genus (Cohen-Bazire, Cohen & Prévot, 1948). They might therefore arise from proteins in the rumen and no doubt some of the normal acids could have a similar origin.

Finally, it is to be expected that the dissimilation of polysaccharides other than cellulose will account for a considerable part of the fatty acids produced in the fermentation of plant structures in the rumen.

SUMMARY

1. The mixture of volatile fatty acids in the rumen of the sheep has been shown to include formic acid, acetic acid, propionic acid, *n*-butyric acid, *iso*-butyric acid, *n*-valeric acid, another valeric acid isomer, caproic acid and an acid which is probably heptoic acid. The proportions in which they are present have been determined.

2. When acetic acid labelled with ^{14}C in the carboxyl group was incorporated in the rumen fermentation *in vitro*, active carbon appeared later in all the higher acids. When labelled propionic acid was included in the fermentation, active carbon appeared in the valeric but not in the butyric acid. The results suggest a synthesis of the higher acids by condensation of the lower ones with 2-C compound in equilibrium with acetic acid. The extent of such syntheses and other possible modes of origin of the fatty acids are discussed.

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THE PHYSIOLOGY OF SEA-URCHIN SPERMATOZOA

THE NATURE AND LOCATION OF THE ENDOGENOUS SUBSTRATE

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INTRODUCTION

Sea-urchin spermatozoa, unlike those of mammals, do not obtain the energy necessary for movement by the enzymatic breakdown of substrates in the external medium. The evidence for this view is twofold: first, these spermatozoa are capable of prolonged activity when suspended in pure sea water; secondly, there is no obvious substrate in the normal external medium, seminal plasma diluted with sea water. Until recently the breakdown of intracellular carbohydrate has been assumed to provide the energy for movement. This assumption is mainly based on measurements of the respiratory quotient by Barron & Goldinger (1941), Hayashi (1946) and Spikes (1948, 1949). These authors do not say in their papers how they measured the respiratory quotient. Dr E. S. G. Barron was kind enough to tell one of us (R.) that he used the direct method; Dr Spikes says in his Ph.D. Thesis (1948) that he also used the direct method. In the absence of further information, Hayashi is assumed to have done the same. Conclusions based on this method, when the medium in which the biological material is suspended is weakly buffered and contains bicarbonate, are of questionable value, as the pH becomes widely different in the two suspensions during measurements. In addition, the possibility that CO_2 may influence sea-urchin sperm metabolism must be considered, if only because the respiration of sea-urchin eggs is higher when measured by Warburg's indirect method than when the direct method is used (Laser & Rothschild, 1939). The only other evidence for carbohydrate being the endogenous substrate is Spikes' statement (1949) that glycogen-like material disappears from the spermatozoa of *Strongylocentrotus purpuratus* and *Lytechinus pictus* during ageing. At the beginning of the one experiment quoted, the content of glycogen-like material was 29·0 mg./g. dry weight of spermatozoa, while after 12 hr. ageing at 19° C., this had fallen to 8·5 mg./g. dry weight. No figures for oxygen uptake during the ageing period are given, though this can be estimated from information in Spikes' Thesis.

Some calculations were recently made regarding the amount of glucose which would have to be present in the middle-piece of a sea-urchin spermatozoon to account for their observed respiration (Rothschild, 1950). The amount was found to correspond to a 20% solution, which seems very high. Subsequently, the carbo-

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hydrate content of spermatozoa and seminal plasma of *Echinus esculentus* was measured (Rothschild & Mann, 1950). The glycogen-like material present in 100 ml. spermatozoa amounted to 110 mg. reducing sugar liberated by acid hydrolysis; of this only 40 mg. was glucose, while the seminal plasma contained little free reducing sugar and no glycogen-like material, though 200 mg. % of some anthrone-reactive carbohydrate, possibly a mucopolysaccharide, were present. The low concentration of sugar in the spermatozoa and the high value required to account for their respiration suggest that carbohydrate is not the principal endogenous substrate of the sea-urchin spermatozoon. The experiments described in this paper were designed to test this possibility. The methods adopted were to measure the oxygen uptake of a suspension of sea-urchin spermatozoa in sea water and the disappearance of glycogen and phospholipid in the same sample, during some 400 min. movement and respiration. The spermatozoa were also examined histochemically and with the electron microscope.

MATERIAL AND METHODS

Material. The semen of *E. esculentus* was obtained by the method of Rothschild & Tuft (1950), and diluted to the required density with sea water containing 50 units penicillin/ml., the penicillin, which has no effect on sea-urchin spermatozoa, being added to delay bacterial contamination (Tyler & Rothschild, 1951). Sperm counts were made with a Fuchs-Rosenthal haemocytometer, the standard error being about 5%.

Manometric procedure. O_2 uptake was measured in Barcroft-Warburg manometers with conical flasks, capacity about 15 ml. 3 ml. of the sperm suspension was placed in the flasks and 0·2 ml. 10% KOH and filter-papers in the centre wells. The gas space contained air. The shaking rate was 90 c.p.m. with a 4 cm. stroke, and the temperature was 20° C.

Phospholipid estimation. 0·07 vol. of 100% (w/v) trichloroacetic acid (TCA) was added to the contents of the manometer flasks and their washings. This was left at 2° C. for 10 min. with intermittent stirring and then centrifuged at 2700 g. for 7½ min. The supernatant was poured off and the precipitate re-suspended in 2 ml. 7% TCA. This was left at 2° C. with intermittent stirring for 10 min., after which the centrifugation procedure was repeated. 4 ml. ethanol was added to the last centrifugate with thorough mixing, and left for 10 min. at room temperature with intermittent stirring. This was centrifuged at 2700 g. for 5 min. and the supernatant collected. The centrifugate was re-suspended in 2 ml. ethanol and left for 10 min. with intermittent stirring. It was then centrifuged as before and the second supernatant was added to the first. 3 ml. of 3:1 ethanol-ether mixture was added to the last centrifugate, which was re-suspended and placed in a water-bath at 62° C. for 2 min. with stirring. The mixture was centrifuged as before and the supernatant was added to the previous two. These were evaporated at 35–45 mm. Hg in a stream of N_2, the temperature not exceeding 45° C. The dry residue was extracted three times with 3 ml. boiling chloroform, some chloroform-insoluble residue being left behind. The chloroform extracts were evaporated to dryness in a boiling water-bath in a stream of air. The lipid residue was combusted in 0·5 ml. 50% H_2SO_4 with

two to three drops of 100 vol. H_2O_2. 2 ml. distilled H_2O was added to the solutions which were then heated for 7 min. in a boiling water-bath. After cooling, the solutions were neutralized to litmus and made up to 10 ml. with distilled H_2O. P determinations were made on 5 ml. aliquots by the method of Fiske & Subbarow (1925). The replicates did not vary by more than 5%.

RESULTS

Phospholipid metabolism and O_2 *uptake.* The results of one experiment, together with certain relevant calculations, are given in Table 1. The figures involving the

Table 1. *Phospholipid metabolism and respiration of sea-urchin spermatozoa* (Echinus esculentus); T °C., 20.

(1) Volume of sperm suspension	3 ml., in sea water containing 50 units penicillin/ml.
(2) Number of spermatozoa	$2{\cdot}67 \times 10^9$ ($8{\cdot}91 \times 10^8$/ml.)
(3) Dry weight of (2)	16·9 mg.
(4) Phospholipid content of (2) at $t=0$	1·105 mg. (mean of three replicates)
(5) Phospholipid content of (2) at $t=420$ min.	0·885 mg. (mean of three replicates)
(6) Phospholipid metabolized in 420 min.	0·220 mg. (−20%)
(7) Theoretical O_2 uptake for combustion of (6)	355 μl.
(8) Actual O_2 consumed by (2)	448 μl. (mean of three replicates)
(9) (7)/(8)	0·79
(10) Phospholipid content, mg./100 ml. fresh semen	737
(11) Phospholipid content, mg./100 mg. fresh spermatozoa (dry weight)	5·7
(12) Phospholipid content, mg./100 ml. seminal plasma	20

dry weight of the $2{\cdot}67 \times 10^9$ spermatozoa in the suspension, i.e. (3) and (11) in Table 1, require explanation. The volume of a sea-urchin spermatozoon is about 18 μ^3 (Rothschild, 1950). 1 ml. of this semen (dry weight 139 mg.), which contained $1{\cdot}78 \times 10^{10}$ spermatozoa, therefore consisted of 0·32 ml. spermatozoa and 0·68 ml. seminal plasma. The dry weight of the latter was 39 mg. making the dry weight of the spermatozoa 113 mg. and of $2{\cdot}67 \times 10^9$ spermatozoa 17 mg. Two assumptions are made in the calculations: first, that the phosphorus content of phospholipid is 4% (Maclean & Maclean, 1927), and secondly, that the combustion of 1 mg. phospholipid involves the disappearance of 1·6 ml. O_2. The complete combustion of 1 mg. carbohydrate is associated with the disappearance of 0·75 ml. O_2.

The results of all experiments are summarized in Table 2.

Lipid cytochemistry. Post-chromated Helly-fixed preparations of fresh and aged spermatozoa were examined unstained and after staining with Sudan Black. No differences in structure or lipid concentration were evident on visual examination, but the greatest concentration of Sudan Black stainable material was present in the middle-piece.

When very dilute sperm suspensions (10^5–10^6/ml.) were aged for 7 hr. and then fixed in formalin (final concentration 2%), marked differences between the middle-pieces of fresh and aged spermatozoa were seen both with the light and the electron

Table 2. *Phospholipid metabolism and respiration of sea-urchin spermatozoa* (Echinus esculentus); T °C., 20.

Pl., phospholipid; Δ, percentage difference in phospholipid content; $R(O_2)$, ratio of theoretical O_2 uptake associated with the observed disappearance of phospholipid, to actual O_2 uptake. The figures in brackets in the column headed 'Exp. no.' are the number of replicates per experiment.

Exp. no.	Time (min.)	mg. Pl./10^{10} spermatozoa	Δ	−ml. O_2/10^{10} spermatozoa	$R(O_2)$
1 (4)	0	4·24	15	1·316	0·78
	515	3·61			
2 (4)	0	4·24	16	1·337	0·83
	465	3·55			
3 (4)	0	4·20	17	1·269	0·92
	390	3·48			
4 (3)	0	4·13	20	1·676	0·79
	420	3·31			
5 (4)	0	3·91	24	1·502	0·99
	395	2·99			

	Arithmetic mean	Standard error
mg. phospholipid per 10^{10} spermatozoa at $t=0$	4·14	0·06
% disappearance of phospholipid per 10^{10} spermatozoa; average incubation time, 437 min.	19·0	2·4
ml. O_2 consumed per 10^{10} spermatozoa; average incubation time, 437 min.	1·450	0·118
$R(O_2)$	0·86	0·04

microscope.* The main differences consisted of an elongation of the middle-piece and a greater separation of it from the head of the spermatozoon.

Carbohydrate metabolism. In spite of the earlier experiments on the carbohydrate content of the spermatozoa and seminal plasma of these sea-urchins (Rothschild & Mann, 1950), we re-estimated the amount of glycogen-like material in a sample of fresh semen, and in an aliquot of the same sample after aerobic incubation, using the same methods as before. 0·15 ml. of fresh semen, sperm density $1{\cdot}90 \times 10^{10}$/ml., contained 0·2 mg. glycogen-like material. After aerobic incubation in the presence of KOH for 390 min. at 20° C., the semen being diluted 1:20 with sea water, the amount of glycogen-like material had fallen to 0·18 mg., a change of about 10%. The complete combustion of 0·02 mg. of this material would involve the disappearance of 17 μl. O_2, whereas the observed consumption of oxygen by the same suspension was 361 μl.

DISCUSSION

Spikes (1948, 1949) found that the total reducing value of a sperm suspension of *Strongylocentrotus purpuratus* fell by 21·3 mg./g. dry weight of semen during 12 hr. ageing at 19° C. The dry weight of 10^8 spermatozoa of this species was 0·35 mg., from which, omitting the small weight contribution of the seminal plasma, it follows that 1 g. of the dried material contained $2{\cdot}86 \times 10^{11}$ spermatozoa. Spikes says that 10^8 spermatozoa consumed some 6 μl. O_2 per hour during periods of the order of 12 hr. $2{\cdot}86 \times 10^{11}$ spermatozoa would therefore consume about 2×10^5 μl. O_2 in 12 hr. The disappearance of 2×10^5 μl. O_2 requires the oxidation

* We are much obliged to Dr J. R. G. Bradfield, Cavendish Laboratory and Department of Zoology, Cambridge, for taking electron micrographs of these spermatozoa for us.

of 268 mg. glucose, more than ten times the observed amount. The conclusion is inescapable that Spikes' experiments do not support his view that the oxidation of intracellular carbohydrate accounts for the oxygen uptake of sea-urchin spermatozoa.

Rothschild & Mann (1950) found 40 mg. glucose/100 ml. of the fresh spermatozoa of *Echinus esculentus*, after hydrolysis with KOH and ethanol precipitation. This means that $2{\cdot}67 \times 10^9$ spermatozoa, the number used in the experiment described in Table 1, contained $1{\cdot}92 \times 10^{-2}$ mg. glucose, the oxidation of which would cause the disappearance of 14 μl. O_2, as compared with the observed uptake of the suspension, 448 μl. O_2. Even if it is assumed, as in the calculations relating to Spikes' experiments, that all the carbohydrate in the semen of *E. esculentus* is available for oxidation, a very improbable assumption, the observed oxygen uptake cannot be accounted for by carbohydrate oxidation. Sea-urchin semen consists of spermatozoa containing 110 mg. % sugar and seminal plasma containing 200 mg. % of an anthrone-reactive carbohydrate. In the experiment in Table 1, 1 ml. semen contained 0·68 ml. seminal plasma and 0·32 ml. spermatozoa. The experimental suspension, which was 3 ml. of semen diluted 1 : 20 with sea water, contained 0·26 mg. carbohydrate, the oxidation of which would cause the disappearance of some 200 μl. O_2, a little less than half the observed amount. The conclusions from these calculations are consistent with the experiment on glycogen metabolism done this year.

Although a number of substances exert a protective effect on sea-urchin spermatozoa when added to the external medium (Rothschild, 1951), there is no evidence that any of them are utilized to a significant extent as substrates, even though some are carbohydrates.

The capacity of sea-urchin spermatozoa to utilize endogenous phospholipid as a source of energy reveals an interesting and previously unnoticed similarity between these spermatozoa and those of mammals. Normally, the latter obtain the energy necessary for movement by the breakdown of the exogenous substrate fructose, secreted into the seminal plasma by the seminal vesicles (Mann, 1949; Mann & Parsons, 1950). Fructolysis is an anaerobic process; consequently, mammalian spermatozoa are capable of sustained movement in the absence of oxygen. But if suspended in a substrate-free medium, they cannot move unless oxygen is available (Lardy & Phillips, 1941). The same applies to sea-urchin spermatozoa (Harvey, 1930), though in this case the external medium, seminal plasma diluted with sea water, differs from that of mammalian spermatozoa in containing no obvious substrate. Lardy & Phillips also showed that bull spermatozoa suspended in sugar-free Ringer phosphate were able to move in the presence of oxygen, and that in these circumstances there was a decrease in their phospholipid content during incubation. One of the main differences between mammalian and sea-urchin semen is therefore that the former normally contains a source of energy outside the spermatozoa which they can use anaerobically, while the latter does not. This does not imply that sea-urchin spermatozoa are devoid of glycolytic enzymes; if, however, they are present, their effectiveness is limited by the lack of utilizable substrate in the external medium and the negligible quantity of carbohydrate inside the spermatozoa.

SUMMARY

1. Sea-urchin spermatozoa (*Echinus esculentus*) contain 4·14 mg. phospholipid per 10^{10} spermatozoa (arithmetic mean of five replicated experiments, standard error 0·06). This amount of phospholipid is about 5·5% of the dry weight of a sea-urchin spermatozoon.

2. The seminal plasma contains very small quantities of phospholipid, about 20 mg./100 ml., less than one-thirtieth the content of fresh semen.

3. When sea-urchin semen was diluted 1:20 with sea water and the spermatozoa incubated aerobically for some 7 hr. at 20° C., phospholipid disappeared. The average disappearance per 10^{10} spermatozoa was 19·0% (S.E. 2·4), while the corresponding oxygen uptake of the same sperm suspensions during the same time was 1·450 ml. (S.E. 0·118). The oxidation of glycogen or glycogen-like material was found to be entirely insufficient to account for the observed oxygen consumption.

4. Assuming that the combustion of 1 mg. phospholipid requires 1·6 ml. oxygen, the ratio of the theoretical oxygen uptake (associated with the observed disappearance of phospholipid) to the observed oxygen uptake was 0·86 (S.E. 0·04).

5. It is concluded that the oxidative breakdown of phospholipid, located in the middle-piece, is the principal source of the energy required for movement.

One of us (R.) is indebted to the Medical Research Council for the provision of a laboratory assistant.

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THE INDUCTION OF REGENERATION IN THE HYDROID *CORDYLOPHORA LACUSTRIS*

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(With Plate 5 and Fifteen Text-figures)

I. INTRODUCTION

The hydroid Coelenterates possess far-reaching powers of regeneration; not only can tissue be replaced but small unspecialized pieces of tissue can become reorganized into the whole organism. The combination of simplicity of structure and extensive regenerative power offers a very favourable subject for studies of development and form regulation. The aim of the present study is to investigate the stimuli initiating, inhibiting and controlling morphogenesis.

The results from previous work which form the background of the present investigation may briefly be stated as follows:

(1) Any healthy unspecialized hydroid tissue can regenerate a hydranth, when in a favourable environment and when free from the dominating influence of hydranths or developing hydranths.

(2) For regeneration to occur, a sufficient supply of oxygen is required (Barth, 1940). There is, however, no evidence to support the theory of Child (1946) and Barth (1940) that oxygen or a gradient of oxygen is a specific 'organizer', or that availability of oxygen is more than one of many conditions necessary for regeneration to occur.

(3) Regeneration can be stimulated and localized by grafting the oral region of the hydranth into unspecialized tissue (Browne, 1909; Mutz, 1930; Rand, Bovard & Minnich, 1926; Goetsch, 1929). This process of induction of development by grafts is investigated further in the present study.

The means by which the induction stimulus is conveyed from the graft to the host is not known. Child's claim that the inducing power of apical tissue depends solely upon a relatively high metabolic rate is difficult to justify experimentally. There is indirect evidence that the hypostome may have a high rate of oxygen consumption; dyes such as methylene blue are reduced more quickly by the hypostome than by other regions (Child, 1946), but there is no evidence that this property is the basis of inducing power; Yao (1945) studied hypostome inductions in *Hydra* and showed that inducing action is not modified by respiratory stimulators or inhibitors.

'Reconstitution masses' formed from dissociated hydroid tissue may regenerate hydranths (Wilson, 1911 and others). Factors determining polarity can therefore be studied in tissue in which the original polarity has been obliterated. Beadle &

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Booth (1938) showed that an oral cone grafted into a reconstitution mass of *Cordylophora lacustris* may induce hydranth formation at the graft site. This work was taken as the starting point for this investigation of the process of induction of hydranth regeneration by oral tissue grafts.

At the outset, Beadle & Booth's experiments were repeated, establishing the inducing power of the oral cone (see § III of the paper). Grafts of other tissues were then made, and it was found that inducing power is not confined to the oral cone but is a property of all regions possessing hydranth differentiation (§ IV). Experiments were then designed to detect any diffusing chemical mediating graft action, but direct close contact between the graft and the host tissue was found to be necessary for the graft to exert its effect (§ V). An account of the histological examination of developing masses is reserved for a later paper, in which it is shown that interstitial cells which accumulate in developing outgrowth tips persist in the adult oral cone, and do not differentiate into regenerant tissues as has previously been assumed. The inducing ability of a graft has been shown to be correlated with the presence of such an interstitial cell accumulation in the graft endoderm.

II. MATERIAL

The brackish water hydroid *Cordylophora lacustris* (Allman) used by Beadle & Booth (1938) proved to be healthier in laboratory conditions than marine hydroids, and has therefore been used throughout the present investigation. The hydroid (see Text-fig. 1) was collected on the River Tamar, near Calstock, Devon, and from wooden structures in the River Ouse, near Downham Market, Norfolk.

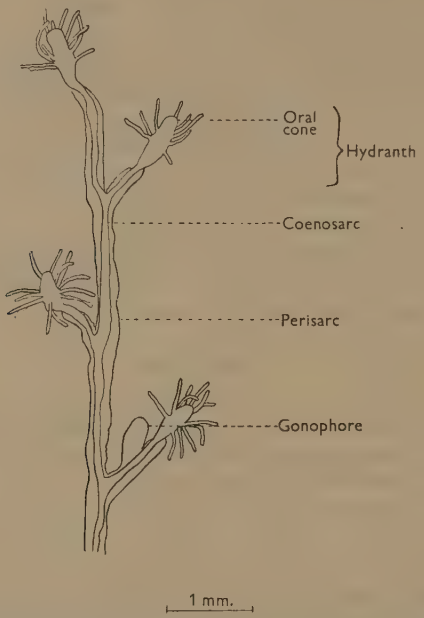
The colonies were kept for several weeks in the laboratory. The culture solution found to be most satisfactory (for the effects of various solutions tried see Singer (1950)) was sea-water diluted 50% with Cambridge river water or with Plymouth tap water. The colonies were placed in an open bowl containing about 2 l. of 50% sea water, air was continuously bubbled through and the water was changed about twice a week. Room temperature varied from 12 to 26° C., but was most commonly between 16 and 19° C. Food was not supplied, but a considerable quantity of prospective food was imported with the hydroid.

Difficulties were caused by seasonal fluctuation in the condition of the hydroid. Summer material (May to August) was abundant in the rivers and regenerated very readily. In winter the hydranths regressed leaving only the basal stolons in the river. Such material may regenerate hydranths in laboratory conditions, but its development is slow and uncertain.

III. RECONSTITUTION MASSES AND ORAL CONE GRAFTS

Beadle & Booth (1938) quote ten experiments which demonstrate that an oral cone grafted into a reconstitution mass of *Cordylophora* may induce hydranth development at the site of grafting. In the present investigation 180 experiments have been performed comparing the development of masses with and without grafts, and data concerning the effect of an oral cone graft have been statistically examined. Sets of experiments have been performed at different times of the year on account of seasonal variations in the rate and type of development.

In the present account 'induction' denotes the stimulation and localization of development by a graft or other agent with the participation of the inducing agent in the development produced. 'Spontaneous' is applied to any regeneration which is not induced by a graft.



Text-fig. 1. *Cordylophora lacustris*, part of a colony. Camera lucida drawing from fixed material.

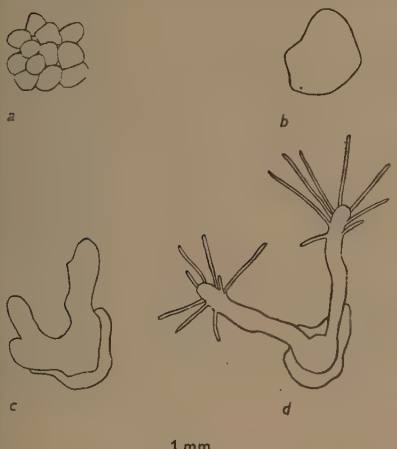
(1) *Method of preparing masses*

Under a binocular microscope the coenosarc of a piece of *Cordylophora* stem is pressed out of the perisarc, using knives made from strips of razor blade mounted on handles. A suitable quantity of tissue is transferred to a watch glass containing some of the culture solution, and the tissue is cut up into fragments about 0·1 mm. in diameter. With glass needles these fragments are piled together into a heap. If an oral cone graft is to be inserted, the oral cone is cut off a hydranth from the colony and placed with the pile of fragments. After half an hour the fragments adhere together, and if an oral cone has been added and has remained in contact with the tissue fragments, it is included in the aggregated mass. The masses are transferred to dishes containing about 60 c.c. of culture solution for two to six masses. The dishes are not aerated and are kept at room temperature, shielded from direct sunlight.

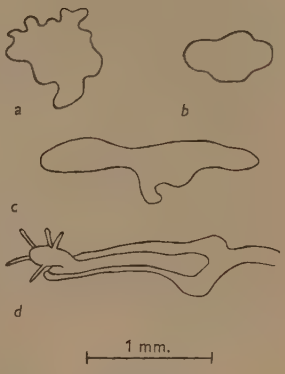
The experiments were paired, one mass with an oral cone graft and one without. Both masses were derived from the same piece of stem and cultured in the same dish, so that the only condition distinguishing the two masses was the presence in one of an oral cone graft.

(2) *The development of masses*

The frequency of regeneration of masses without grafts is given in Table 1. The development of masses is illustrated in Text-figs. 2 and 3 and Pl. 5. As described by



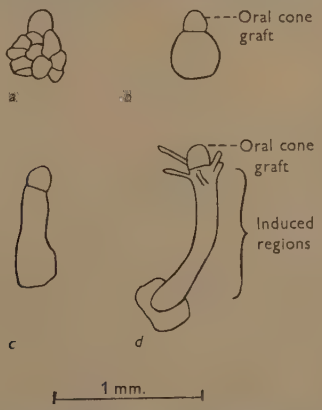
Text-fig. 2.



Text-fig. 3.

Text-fig. 2. The development of a mass without a graft. (*a*) Pile of tissue fragments. (*b*) Aggregated mass, 21 hr. (*c*) Outgrowths and perisarc, 70 hr. (*d*) Hydranths, 142 hr. Camera lucida drawings from living material, except (*a*), diagrammatic.

Text-fig. 3. Stolonic development of a mass without a graft. (*a*) Aggregate mass, 1 hr. (*b*) Stolons appearing, 24 hr. (*c*) Stolons, 46 hr. (*d*) Hydranth budded off from one stolon, shrinkage of the other, 117 hr. Camera lucida drawings from living material.



Text-fig. 4. The development of a mass with an oral cone graft. (*a*) Mass fragments with oral cone graft. (*b*) Aggregated mass with graft, 22 hr. (*c*) Induced outgrowth, 46 hr. (*d*) Induced hydranth, 169 hr. Camera lucida drawings from living material, except (*a*), diagrammatic.

Beadle & Booth (1938), the tissue fragments aggregate into a mass, and the ectoderm and endoderm move into their normal relative positions as shown when a more transparent region can be distinguished at the boundary of the mass. Perisarc is laid down and becomes apparent when the tissue grows out from the original mass to form outgrowths or stolons. A mass may develop either by producing outgrowths which become directly transformed into hydranths at the tip, or by producing stolons which are attached to the substratum along their length with perisarc covering the tip. Stolons may bud off hydranths laterally at a point near the tip. Stolonic development has been found in the present investigation to be frequent following a rise in temperature, at any season of the year (see discussion).

In masses with an oral cone graft, an outgrowth is normally (see Table 1) induced beneath the oral cone, and this outgrowth differentiates into the parts of the hydranth basal to the oral cone, the graft remaining attached as the oral cone of the induced hydranth (see Text-fig. 4 and Pl. 5). Oral cone grafts do not induce the stolonic type of development.

An oral cone cultured in isolation does not regenerate any tissue, except occasionally one or two tentacles. Therefore, the action of a grafted oral cone is a true example of induction, the stimulation and localization of development without the participation of the inducing agent. That the graft and host tissue remain distinct is shown in Pl. 5.

(3) *Results of comparing the development of masses with and without grafts*

(i) *Oral cone grafts induce hydranth formation.*

Table 1. *The number of masses without grafts which regenerated spontaneously, and the number of inductions by oral cone grafts, out of* 180 *experiments*

	No graft	Development induced by a graft
Mass died	55	36
Graft not taken	—	16
Graft absorbed	—	34
Outgrowths not hydranths	39	29
Hydranths	86	64
Graft attached to surface of mass, no induction, development elsewhere	—	1
Total	180	180

Summary of Table 1. (*a*) Out of 180 masses without grafts 125 regenerated (70%). (*b*) Out of 180 masses with oral cone grafts, 128 regenerated with the graft included in the mass (71%).

Out of the 128 successful grafts referred to in (*b*):

Graft attached to the mass surface, development induced at the site of grafting, 93 (72·7%).

Graft absorbed into the mass, no induction, 34 (26·6%).

Graft attached to the mass surface, no induction, 1 (0·7%).

Apart from the minority of experiments in which the graft was absorbed into the mass, an oral cone successfully grafted to a healthy mass produced induction in every experiment except one; the circumstances causing this result are unknown, but it is shown by the other data to be quite exceptional. These results establish the regular production of hydranth induction by oral cones grafted to masses.

(ii) *Oral cone grafts accelerate hydranth formation*

The rate of regeneration varies with the temperature at which the mass is made and cultivated, development being faster at higher temperatures. There are also seasonal differences in the rate of regeneration which are apparently independent of temperature. In Table 2, therefore, the average rate of development is calculated for four different months separately.

Table 2. *The rate of development of masses with and without grafts. The figures represent the average interval, in hours, between the formation of a mass and the appearance of outgrowths or hydranths. The appearance of tentacle buds is the criterion of hydranth formation. Development times are necessarily only approximate.*

Month	T° C.	No. of exps.	No graft		Induced by graft	
			Outgrowths (hr.)	Hydranths (hr.)	Outgrowths (hr.)	Hydranths (hr.)
July 1947	18–21	20	34	71	25	48
Feb. 1949	13–17	50	97	174	67	116
Oct. 1949	15–20	28	44	78	39	64
Jan. 1950	14–17	10	127	187	68	116

Similar results were obtained by Beadle & Booth, but not in sufficient number for statistical treatment. For the present results, summarized in Table 2, Dr D. A. Sholl of the Anatomy Department, University College, London, has very kindly examined statistically the mean differences between the time of appearance of induced and spontaneous development:

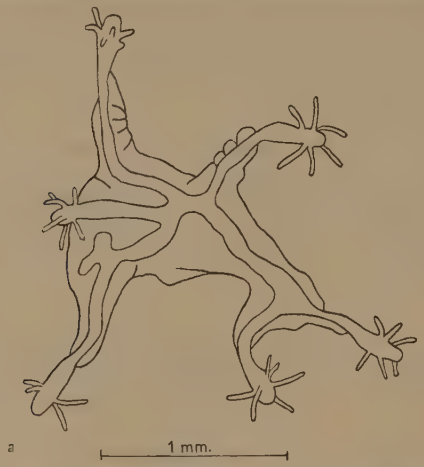
For the winter material (February 1949 and January 1950) the formation of induced outgrowths and hydranths is significantly (on a 2% level) faster than the formation of spontaneous outgrowths and hydranths. These results establish that oral cone grafts not only localize development but may also accelerate it.

For the summer material (July 1947) the formation of induced hydranths is significantly (on a 2% level) faster than the formation of spontaneous hydranths, but the difference is not significant on a 5% level for the experiments performed in October 1949. The acceleration of outgrowth formation has not been established statistically for either of these sets of experiments. This seasonal difference exists partly because, for a given frequency of examination, differences in the rate of regeneration are less readily detected in quickly developing material, and partly because in winter conditions the effect of a graft is more pronounced. Beadle & Booth (1938) obtained inductions by grafts in conditions when masses without

grafts did not regenerate at all, by using culture solutions deficient in certain ions and by lowering the temperature.

(iii) *Oral cone inductions inhibit spontaneous regeneration*

Chalkley (1945) reported that in *Hydra* the number of hydranths regenerated by a mass was proportional to the volume of tissue, but in the present investigation it was found that while the size of the mass influenced the size of the hydranths regenerated, the number of outgrowths and hydranths produced depended upon the



Text-fig. 5. Mass with many hydranths regenerated. Camera lucida drawing from fixed material.

condition of the tissue and not upon its volume. Masses made between December and May rarely regenerate more than one hydranth, while the summer masses regenerated two or three. Masses made from loosely constructed tissue produced a greater number of hydranths (see Text-fig. 5).

The number of outgrowths and hydranths regenerated by a mass is influenced by the presence of an oral cone graft:

Masses without grafts:

78 out of 91 regenerated more than one outgrowth (85·7%).
26 out of 91 regenerated more than one hydranth (28·5%).

Masses with inductions by oral cone grafts:

27 out of 83 regenerated outgrowths additional to the induction (32·5%).
2 out of 83 regenerated hydranths additional to the induction (2·4%).

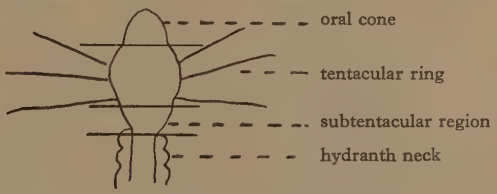
These results show that in the presence of an induction by an oral cone graft, spontaneous regeneration by the mass is less frequent than in masses without grafts.

The results of comparing the development of masses with and without grafts establish the inducing activity of oral cone grafts, and show that oral cone grafts not

only localize development but also accelerate it, and inhibit the spontaneous regeneration of the mass. The effect of grafting into masses tissue other than the oral cone was next investigated.

IV. GRAFTS OF REGIONS OTHER THAN THE ORAL CONE

Previous authors have recorded inducing power only in the oral region of hydroids. Beadle & Booth (1938) stated that a grafted piece of gonophore does not influence the regeneration of a *Cordylophora* mass. No other work on grafting tissue other than the oral cone has been recorded for *Cordylophora*. In *Hydra*, Browne (1909), Mutz (1930) and Yao (1945) found that inducing power is confined to the peristome (hypostome), and that any other region when grafted is absorbed into the host without inducing action. Browne (1909) states that in *Hydra* grafted tentacles do not induce development, but this result is based on only eight successful grafts. Child (1932) found that pieces of the distal region of a *Corymorpha* stem when grafted into another stem become reorganized into hydranths which exert dominance over the host. There was no evidence of induction by these grafts however, nor did a graft exert dominance without becoming reorganized into a hydranth.



Text-fig. 6. The regions of the hydranth.

In the present investigation the following regions of Cordylophora have been grafted into reconstitution masses:

(1) *The regions of the differentiated hydranth.* Oral cone, tentacular ring, subtentacular region, hydranth neck, pieces of tentacle.

(2) *The rudiment of the differentiated hydranth.* Tips of outgrowths developing from masses.

(3) *Regions other than the differentiated hydranth.* Pieces of stem coenosarc, stolon tips.

(1) *The regions of the hydranth*

Experiments

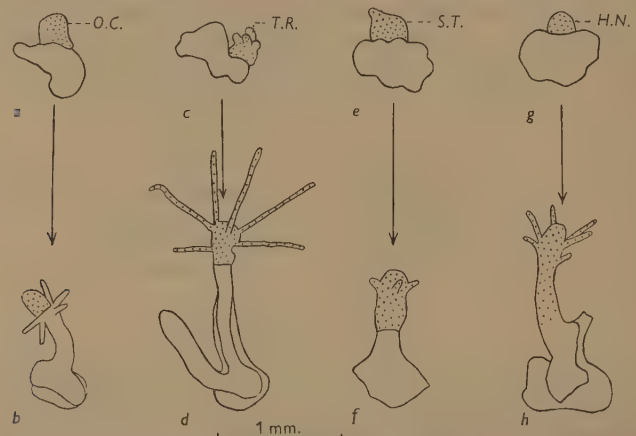
Hydranths were cut as indicated in Text-fig. 6, and the separate regions grafted with their more basal surface against the mass. Tentacles were removed from tentacular ring grafts as their movements otherwise dislodge the graft. The perisarc was removed from hydranth neck grafts. Pieces of tentacle were difficult to graft; a graft can only become attached to a mass before the cut surface of the graft becomes covered with ectoderm, and with a tentacle the area of the cut surface is very small and healing is very rapid. Out of forty-four attempts, fourteen were successful (see Table 3). To act as controls, masses without grafts and masses with oral cone

grafts were made at the same time and cultured in the same dish as masses with grafts of other hydranth regions.

Table 3. *The results of grafting different regions of the hydroid to reconstitution masses.*

Graft	Graft not taken	No. of exps. with successful grafts	Mass died	Graft absorbed	Induction
Oral cone	8	30	1	1	28
Tentacular ring	10	30	8	6	16
Subtentacular region	11	32	3	10	19
Hydranth neck*	2	31	4	7	7
Tentacle	30	14	3	4	7
Outgrowth tip	20	35	0	22	13
Stem coenosarc	3	43	0	43	0

* See text for the effect of thirteen of these experiments.

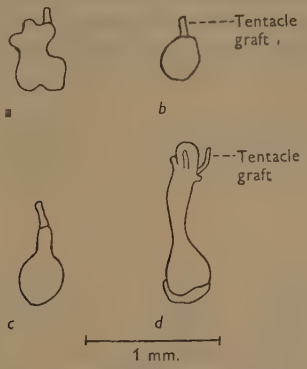


Text-fig. 7. Mass with grafted oral cone (*a*, *b*), tentacular ring (*c*, *d*), subtentacular region (*e*, *f*), and hydranth neck (*g*, *h*). Before development (*a*, *c*, *e*, *g*) and after development (*b*, *d*, *f*, *h*). Graft tissue stippled. Camera lucida drawings from living material.

Results

(i) *The occurrence of induction.* The results summarized in Table 3 establish that the inducing power of the oral cone is shared by basal hydranth regions and by pieces of tentacle. Induced outgrowths may be produced by tentacular ring grafts, subtentacular region grafts and pieces of tentacle, as by oral cone grafts. A tentacular ring graft becomes reorganized to regenerate an oral cone out of the graft tissue, and a subtentacular region graft regenerates an oral cone and a tentacular ring. Parts of the hydranth basal to the graft are induced to develop out of the mass tissue (see Text-fig. 7). In the completed hydranth the graft and host material can be distinguished by a difference in transparency (see Pl. 5 *i*, *j*). Tentacle grafts induce the whole hydranth including the oral cone to develop from the mass tissue (see Text-fig. 8).

Hydranth neck grafts may become the site of hydranth formation. In seven experiments (see Table 3) the graft gave rise to the hydranth, and a stem was induced to develop from the mass. In nine experiments, however, a hydranth was formed by regeneration of graft tissue without the participation of the mass. In four experiments the hydranth neck graft remained attached to the mass without regeneration or induction, while the mass regenerated hydranths elsewhere. The inducing power of the hydranth neck, tissue at the boundary of the differentiated hydranth, is evidently relatively weak.



Text-fig. 8. The development of a mass with a tentacle graft. (*a*) Aggregated mass with graft. (*b*) Mass with graft at spherical stage, 20 hr. (*c*) Induced outgrowth, 96 hr. (*d*) Hydranth induced by tentacle graft, 168 hr. Camera lucida drawings from living material.

(ii) *The rate of development.* The average interval between setting up the mass and graft and the appearance of induced outgrowths was as follows:

Oral cone graft	53 hr. (average of 28 inductions).
Tentacular ring graft	49 hr. (average of 16 inductions).
Subtentacular region graft	57 hr. (average of 19 inductions).
Hydranth neck graft	56 hr. (average of 12 inductions).
Tentacle graft	56 hr. (average of 7 inductions).
Mass without graft, interval before appearance of spontaneous outgrowths	76 hr. (average of 20 masses).

These results show that inductions by all hydranth regions develop at the rate characteristic of oral cone inductions rather than of spontaneous outgrowths.

(iii) *The inhibition of spontaneous development.* In the presence of development induced by any hydranth region graft, spontaneous regeneration is inhibited. Hydranths derived from hydranth neck grafts did not inhibit spontaneous regeneration in those experiments where development was not induced in mass tissue.

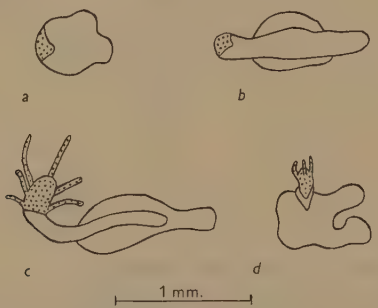
(iv) *Oral cone regeneration by the graft.* Induction by tentacular grafts, subtentacular region grafts and hydranth neck grafts is accompanied by the regeneration of an oral cone by the graft. The inducing power of these grafts might therefore be

attributed to the presence of an oral cone. However, the induced outgrowth may appear before the graft regenerates an oral cone. Grafted pieces of tentacle are unable to regenerate any other tissue; there is no oral cone structure in the inducing graft but the oral cone of the induced hydranth is derived from mass tissue. It is clear, therefore, that inducing power is not a property peculiar to the oral cone.

(2) *The tips of outgrowths from masses*

Experiments

The tips of outgrowths spontaneously regenerated by masses were cut off and used as grafts. To distinguish these small grafts, vital staining with neutral red or Nile blue was used. Comparison of experiments with stained and unstained grafts showed that the staining did not influence the results.



Text-fig. 9. Mass with outgrowth tip graft. Graft tissue stippled. (*a*) Aggregated mass with graft. (*b*) Induction by outgrowth tip graft, 47 hr. (*c*) Induced hydranth, 139 hr. (*d*) Outgrowth tip reorganized in isolation, from another mass. Camera lucida drawings from living material.

Results

Outgrowth tip grafts may become reorganized into apical hydranth tissue and induce structures more basal to develop from the mass (see Text-fig. 9). The proportion of the grafts which produced induction is shown in Table 3. The extent to which the induced hydranth was composed of graft tissue varied, and appeared to depend upon the size and age of the graft. Inductions by outgrowth tip grafts were similar to inductions by hydranth regions in rate of development and inhibition of spontaneous regeneration. Induced outgrowths appeared either before or after the differentiation of the graft. In six examples where the graft failed to take, the isolated outgrowth tip differentiated into a small complete hydranth (see Text-fig. 9*d*).

(3) *Regions other than the differentiated hydranth*

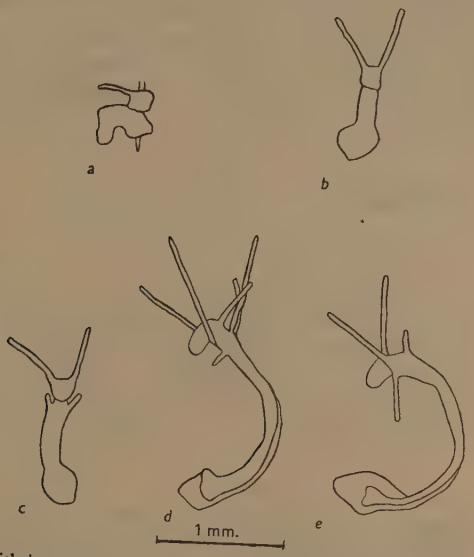
(i) *Stem coenosarc grafts*

Pieces of intact stem coenosarc, similar in size to oral cones, were grafted to masses to find whether induction could be produced by unspecialized tissue differing from the mass fragments only in size. To distinguish the graft, it was stained with neutral red or Nile blue in some of the experiments.

Forty-six experiments were performed, in forty-three of which the graft took. In no case was any induction observed: the graft was absorbed into the mass without any apparent influence on the regeneration of the mass. Table 3 compares these results with those of grafting regions of the differentiated hydranth.

(ii) *Stolon tip grafts*

Stolon tips cannot easily be removed from the perisarc which surrounds them, and even when the tissue was extracted and grafted to a mass it secreted more perisarc and became separated from the mass fragments. No induction nor graft differentiation has been observed.



Text-fig. 10. Mass with inverted oral cone graft. (*a*) Inverted oral cone graft, tentacle at free end. (*b*) Induced outgrowth, 69 hr. (*c*) Induced tentacles, 93 hr. (*d*) Oral cone graft turning to normal orientation, 165 hr. (*e*) Induced hydranth with normally orientated oral cone, 216 hr. Camera lucida drawings from living material.

The results of these grafting experiments suggest that inducing power depends upon the presence of hydranth differentiation rather than upon any other factor (see discussion). This conclusion was further tested by grafting to masses differentiated tissue which was abnormally orientated or fragmentary:

(4) *Inverted oral cone grafts*

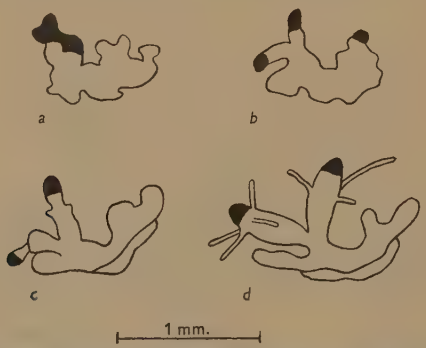
Beadle & Booth (1938) state that some of their oral cone grafts appeared to be inverted, with the mouth against the mass and the tentacular end free, but that they found no way of controlling the orientation of a graft. Using the spearing technique described in § V (see below), it was possible in the present investigation to graft inverted oral cones on to masses.

In seven out of eleven experiments, inverted oral cone grafts produced induction. After induced tentacles appeared, in several experiments the graft righted itself to achieve its normal orientation (see Text-fig. 10).

(5) *Fragments of oral cones*

Oral cones which had been stained with neutral red or Nile blue were chopped into small fragments, and these graft fragments were placed with mass tissue fragments.

Out of twenty-three experiments, the oral cone fragments induced development of the more basal regions of the hydranth in ten, and were absorbed into the mass without induction in eleven. The basal part of the oral cone was formed out of the induced structure (see Text-fig. 11*c*) or in other cases the oral cone fragment constituted the entire oral cone of the induced hydranth. In some experiments several separate inductions were produced from one mass by different oral cone fragments (see Text-fig. 11).



Text-fig. 11. Mass with oral cone fragments grafted. Graft tissue black. (*a*) Aggregated mass with graft. (*b*) Inductions by the separate fragment grafts, 23 hr. (*c*) Differentiation of the induced outgrowths, 47 hr. (*d*) Induced hydranths, 73 hr. Camera lucida drawings from living material.

That inverted oral cones and fragments of oral cones have inducing action similar to that of normally orientated intact oral cones supports the conclusion that inducing action is a property of any piece of tissue which is in the state of hydranth differentiation.

V. EXPERIMENTS DESIGNED TO DETECT A DIFFUSING CHEMICAL INDUCTOR

Three types of experiments were performed to test whether the induction of hydranth regeneration by an oral cone graft is mediated by a diffusing chemical inductor:

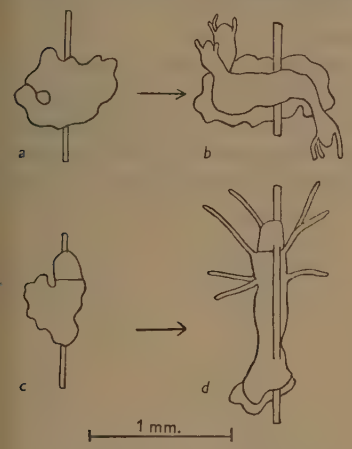
(1) The interposition of an agar block between an oral cone graft and a mass.

(2) The grafting to a mass of an agar block previously in contact with oral cones.

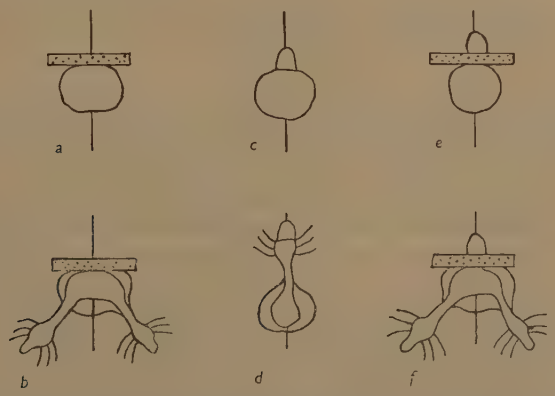
(3) The interposition of cigarette paper between an oral cone graft and a piece of hydroid stem.

(1) *Use of agar barriers*

Went (1935) used agar blocks interposed between shoot tip and stem in the classical experiments demonstrating the existence of plant growth hormones. The characteristic shoot tip effects were transmitted through the agar, indicating that the shoot tip effects were mediated by a diffusing substance. To apply this technique to the present problem, a means had first to be found of holding the tissue and agar together. It was found that an oral cone can be joined to a mass by spearing with a fine glass fibre (see Text-fig. 12). Masses without grafts which are speared with a glass fibre regenerate hydranths laterally and are apparently uninfluenced by the presence of the spear (see Text-fig. 12*b*). An oral cone speared on to a mass may induce an outgrowth to form at the point of grafting, around the glass fibre. As the outgrowth develops the tissue frees itself from the glass and a normal induced hydranth is formed (Text-fig. 12*d*). The proportion of grafts successfully included in the aggregation of the mass fragments is lower than for the unspeared grafts, but in all other respects speared masses develop in a similar manner and at a similar rate to unspeared masses. Spearing of grafts was first performed by King (1902) working on *Hydra*.



Text-fig. 12. The spearing technique. (*a*), (*b*) Speared mass, before and after development. (*c*), (*d*) Speared mass with oral cone graft, before and after development. Camera lucida drawings from fixed material.

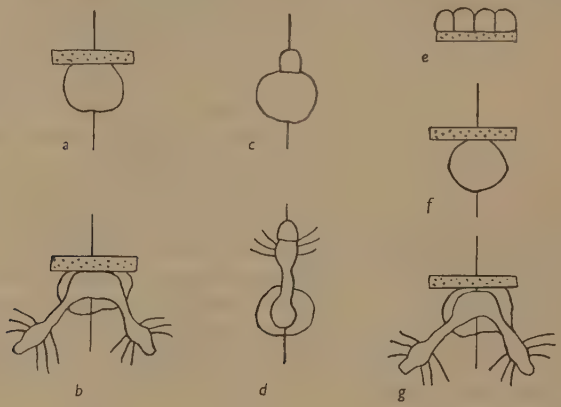


Text-fig. 13. Agar barriers. (*a*), (*b*) Agar speared to a mass (control). (*c*), (*d*) An oral cone speared directly to a mass (control). (*e*), (*f*) An oral cone speared to a mass with an agar barrier. Before (*a*, *c*, *e*) and after (*b*, *d*, *f*) development. Diagrammatic.

The spearing technique made it possible to join an oral cone to a mass with an agar barrier between them (see Text-fig. 13). In twelve experiments, development was indistinguishable from that of control masses against agar without an oral cone. No influence of the oral cone through the agar could be detected.

(2) *Grafts of agar previously exposed to oral cones*

These experiments were modified from those in which Mangold (1932) claimed to demonstrate the neural inductor in the amphibian embryo. A number of oral cones were piled on to a thin slice of agar, and the surrounding water drawn off so that the oral cones were held down against the agar by the surface film. After periods varying from four to twenty-four hours, all the fragments of oral cone were carefully removed from the agar and the agar speared to a mass (see Text-fig. 14).



Text-fig. 14. (*a*), (*b*), Agar speared to a mass (control). (*c*), (*d*) An oral cone speared directly to a mass (control). (*e*) Oral cones set on agar. (*f*), (*g*) Agar from (*e*) speared to a mass. Before (*a*, *c*, *f*) and after (*b*, *d*, *g*) development. Diagrammatic.

In twelve experiments, the regeneration of the masses in contact with the treated agar was indistinguishable from that of control masses against untreated agar. Eight out of twelve control oral cone grafts produced induction.

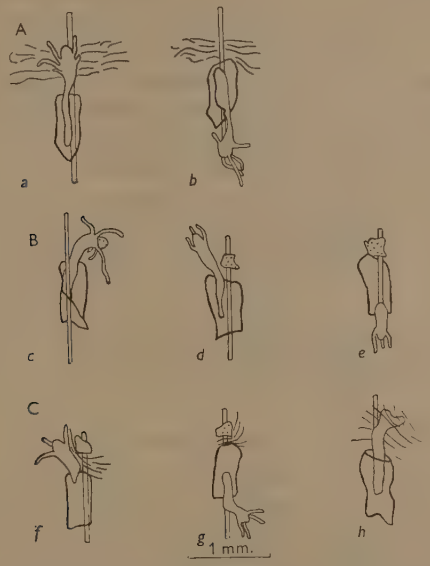
(3) *Experiments using a cigarette paper barrier*

Cigarette paper was substituted for agar because it provides a much thinner barrier, and the holes between the fibres should canalize the diffusion of any chemical inductor. Intact pieces of stem enclosed in perisarc were substituted for masses in the hope of obtaining closer contact between the barrier and the host tissue. That cigarette paper does not form an impassable barrier to growth was shown by two examples in which outgrowths formed under the paper pushed between the fibres and grew up on the other side (Text-fig. 15*h*).

Sixty-one oral cones have been grafted to stem-pieces with cigarette paper barriers, and there was no indication of induction in any. Stem pieces separated from an oral

cone by cigarette paper regenerated spontaneously at either end, as did control pieces speared to cigarette paper with no oral cone (see Text-fig. 15*a*, *b*, *f*, *g*). The frequency of regeneration at either end of the piece depended upon its original polarity in the stem, and was not influenced by the presence of the oral cone.

Out of eighty oral cones grafted directly to stem pieces, with no cigarette paper barriers, forty-three produced induction (see Text-fig. 15*c*). In a further seventy-eight experiments, the oral cone failed to become attached to the cut surface; the grafting was less easy than with reconstitution masses. The oral cone may however remain in position at the cut surface, while the stem tissue regenerates uninfluenced by the oral cone (Text-fig. 15*d*, *e*).



Text-fig. 15. Cigarette paper barriers. A. Cigarette paper speared to a stem piece (control): (*a*) Spontaneous hydranth under the paper. (*b*) Spontaneous hydranth at the remote end. B. An oral cone speared directly to a stem piece (control): (*c*) Induced hydranth. (*d*) Graft excluded, spontaneous hydranth beside it. (*e*) Graft excluded, spontaneous hydranth at the remote end. C. An oral cone graft with a cigarette paper barrier: (*f*) Spontaneous hydranth under the paper. (*g*) Spontaneous hydranth at the remote end. (*h*) Outgrowth through the paper. Camera lucida drawings from fixed material, except (*a*), (*d*), (*f*) which are diagrammatic. Grafts are stippled.

In the experiments which have been described no oral cone action has been transmitted through an agar or cigarette paper barrier, and no evidence of a diffusing inductor obtained. The need for direct close contact between the graft and host if the graft is to be effective is further shown in those experiments where a piece of stem regenerated unaffected by an oral cone which remained in position at the cut surface without becoming attached to the tissue.

Further experiments were performed to test whether chemical constituents of oral cone tissue could produce induction.

(4) *Grafting killed oral cones*

Attempts were made to graft to masses oral cones previously killed by boiling, 70% alcohol, formalin or Zenker's fluid. Controls were set up of grafts of stem coenosarc similarly killed, and living grafts of oral cones and stem coenosarc.

In all experiments where killed tissue was grafted the living tissue, even when affixed by a glass spear, shrank away from the graft and regenerated independently of it. Tissue killed by all the methods listed quickly degenerated on being returned to the culture solution. The control grafts of living tissue developed normally.

(5) *Grafting macerated oral cones*

Oral cones stained with neutral red or Nile blue were chopped up and the tissue pulled apart with needles so that the histological structure of the small pieces was destroyed. The macerated tissue was grafted to a mass.

Macerated oral cone tissue had no inducing action in any of the nineteen experiments, while nine out of sixteen intact oral cones grafted as controls produced induction. In five experiments however, the macerated tissue recombined into oral cone fragments, and these fragments then induced basal hydranth regions to develop from the mass.

These results show that while small fragments of oral cone tissue possess the inducing properties characteristic of the entire structure, no inductions have been obtained by grafting tissue further dissociated.

VI. DISCUSSION

(1) *Tissue with hydranth differentiation has inducing power*

An oral cone grafted to a reconstitution mass induces hydranth regeneration at the point of grafting. This property has been shown to be shared by grafts of any other region of the hydranth, and by the tips of mass outgrowths of the type which directly differentiate into hydranths. Induction may be produced even by grafting a small piece of tentacle; this has not previously been recorded for any hydroid. Regions of the hydroid which are not a part of nor a rudiment of the differentiated hydranth, pieces of unspecialized coenosarc and tips of stolons, have no inducing power when grafted to a mass.

Tentacles are offshoots of the main axis of the hydranth, and tentacles may induce the development of a structure more apical in the axis: the oral cone. These facts are difficult to reconcile with Child's theory that inducing activity is a property of apical structures because these possess a high metabolic rate relative to tissue more basal. Child's theory that apical regions have a relatively high metabolic rate rests largely on indirect evidence, such as the differential reduction of dyes and the differential speed of reaction to cyanide. Whether or not apical tissue has a high metabolic rate, no evidence exists to prove that a high metabolic rate confers inducing properties.

The results of the present investigation associate inducing properties with the presence of differentiation rather than with a simple quantitative variable.

(2) *Inducing grafts initiate growth and differentiation*

Induction by a part of the hydranth as small as a piece of tentacle illustrates clearly that the graft provides an initial stimulus which localizes growth and differentiation in the host tissue rather than 'imprinting' its own character on this tissue; the action of a tentacle graft is not confined to induction of tentacle differentiation, nor even of hydranth differentiation, but at the site of the graft an outgrowth is first formed. The interval before differentiation sets in is very variable, whether measured in time or as the length of the undifferentiated outgrowth.

Li & Yao (1945) found that while the developing hypostome grafted into a *Hydra* induces the formation of outgrowths which later differentiate, adult hypostome grafts induce differentiation at the outset. This distinction between grafts of adult and young hydranth tissue was not found however in the present investigation of *Cordylophora* reconstitution masses; both young and adult hydranth tissue induces outgrowth formation before hydranth differentiation.

(3) *The inhibition associated with induction*

The induction of development by a graft has been shown to inhibit the spontaneous regeneration of a mass. The induction and inhibition effects are inseparable; all grafts which produce induction produce inhibition, and grafts which are absorbed into a mass without inducing action do not inhibit its regeneration. Grafting the hydranth neck, tissue which is at the boundary of hydranth differentiation and is variable in its effect, clearly demonstrates the inseparability of induction and inhibition. If the graft produces induction, spontaneous regeneration of the mass is inhibited. If the graft does not induce regeneration out of mass tissue, even though the graft tissue differentiates into a hydranth, the mass may regenerate supernumerary hydranths. It is not the presence of the graft, whether in the form in which it is grafted or after hydranth regeneration, which inhibits spontaneous regeneration, but the production of induction. The inhibition effect is an aspect of the induction process rather than a phenomenon associated with any other property of the grafted tissue.

Spiegelman (1945) attributed the dominance of hydranths and developing hydranths in hydroid colonies to a competitive relationship. The example of hydranth dominance provided by the inhibition of spontaneous regeneration by a graft with inducing action is most readily explained in terms of competition between possible sites of development for cells or for substances required for development. That development induced by a graft is normally successful in competition with potential spontaneous regeneration is probably because the graft not only localizes development but also accelerates it.

(4) *Stolonic development*

Masses may regenerate by stolon production instead of or as well as by outgrowths which directly differentiate into hydranths. Stolons appear to be inhibited

hydranths, outgrowths in which hydranth differentiation is deferred. Stolonic development has been found in the present investigation to be frequent following a rise in temperature, at any season of the year (cf. Berrill (1949) for colonies of *Aselomaris* and *Bourgainvillea* growing in natural conditions). Beadle & Booth (1938) record only one example of stolonic development out of twenty *Cordylophora* masses, probably because the masses were cultivated at a constant temperature (16–17° C.).

It has been found also that masses derived from colonies bearing ripe gonophores have a particularly high frequency of stolonic development, and with this tissue there is also a tendency for oral cone grafts to be absorbed rather than to induce development. The correlation between reproductive activity and stolon production was recorded by Peebles (1931) for *Tubularia*. Ripe gonophores may be found on *Cordylophora* colonies at any period between May and September. Possibly the stimulus to stolon production supplied by a rise in temperature may be related to changes initiating gonophore development.

(5) *The relation of induction to the initiation of development in the absence of a graft*

Masses can regenerate hydranths in the absence of any induction stimulus. While induction by grafted differentiated tissue is unlikely to be entirely different from the naturally occurring developmental process, the only evidence of the inducing action of the oral region is derived from grafting experiments.

Histological work to be described in a later paper has revealed an accumulation of interstitial cells in the endoderm of developing outgrowth tips. These interstitial cells do not differentiate into ectoderm and endoderm but persist unchanged in the adult oral cone. Regions of growth which do not contain the oral cone rudiment, such as stolon tips, have no interstitial cell accumulations. Regions undergoing differentiation which do not contain the oral cone rudiment, such as induced outgrowths, have no interstitial cell accumulations.

The interstitial cell accumulation which represents the oral cone rudiment is not however laid down at the beginning of outgrowth formation, but appears about twenty-four hours later. Therefore the initial localization of 'spontaneous' outgrowths must be attributed to some other cause. The tissue has been chopped up and the original polarity obliterated, and it is all equally exposed to the environment. Child (1928) stated that hydranths develop at the top surface of *Corymorpha* masses where the oxygen tension is presumed to be greatest. This observation does not apply to *Cordylophora* masses. Variation in the sizes of the pieces of tissue composing the mass is unlikely to localize regeneration, since grafting a piece of stem coenosarc the same size as an oral cone does not determine the site of regeneration.

Holtfreter (1947) has shown that cell damage is a sufficient stimulus to initiate neural differentiation in amphibian embryonic ectoderm, and suggests a parallel between parthenogenesis, neural differentiation and malignant growth, that all these phenomena may be induced by unspecific stimuli causing damage to the cell membrane (Holtfreter, 1948). The general association of regeneration with damage suggests that it also may be initiated by a stimulus associated with damage to cells. In hydroid reconstitution masses, the tissue has been chopped up into small pieces.

This considerable 'damage' may stimulate the regeneration of the mass, but since it extends to the whole of the tissue it is difficult to see how the damage can localize regeneration. After the mass tissue has rounded up, local damage to the surface does not initiate development at that point. Grafts of foreign substances to hydroid masses may therefore be very much more informative than in investigation of embryonic neural induction, where induction by any artificially applied substance may be attributed to cell damage.

The stimulus initiating localized 'spontaneous' outgrowths remains unknown. After development has been initiated, an interstitial cell accumulation shows that the oral cone rudiment appears in the outgrowth tip, and induced and spontaneous hydranth regeneration may be similarly controlled. Induction by a graft is, however, a more powerful stimulus to development than occurs in spontaneous regeneration; development is faster when it is induced by a graft, and induced development may occur in conditions where spontaneous development is suppressed.

(6) *The mediation of the induction stimulus*

No evidence has been obtained of a diffusing chemical mediating hydranth induction. Induction was produced only when graft tissue was in direct close contact with the host, and only when the grafted differentiated tissue had a part of its histological structure complete; grafted macerated or killed tissue did not produce induction. That grafts which are broken down and absorbed into a mass do not localize regeneration further suggests that induction is produced by differentiated tissue in its normal form rather than by the constituents of differentiated tissue.

As will be shown in a later paper, a correlation has been established between the inducing ability of differentiated tissue and the presence of a dense accumulation of interstitial cells in the endoderm. That interstitial cells are found to be particularly rich in ribonucleic acid may or may not be significant in relation to inducing power.

SUMMARY

1. Beadle & Booth's work on grafting oral cones into reconstitution masses of *Cordylophora lacustris* has been repeated, confirmed and extended. Oral cone grafts induce the development from mass tissue of hydranth regions basal to the oral cone, and other regeneration of the mass is inhibited. Except in the most quickly developing material, induced development is significantly faster than 'spontaneous' development.

2. Inducing action when grafted to a mass is not peculiar to the oral cone but is a property of all tissue with hydranth differentiation (the tentacular ring, subtentacular region, hydranth neck and even pieces of tentacle), and of tissue containing the rudiment of hydranth differentiation (the tips of outgrowths developing from masses). Tissue which does not possess hydranth differentiation (unspecialized stem coenosarc and stolon tips) has no inducing activity but is absorbed into the mass without influencing its regeneration.

3. Grafts of basal hydranth regions and outgrowth tips normally regenerate oral cones out of graft tissue, but induction may occur even when no oral cone is

regenerated by the graft. Tentacle grafts do not regenerate an oral cone; the oral cone of the induced hydranth develops from mass tissue. Inducing power therefore does not depend upon the regeneration by the graft of an oral cone.

4. Grafts of inverted oral cones or fragments of oral cones may produce induction. That inducing activity does not depend on the size, orientation or completeness of the grafted differentiated tissue further shows that it is the presence of hydranth differentiation in tissue, rather than any other factor, which confers inducing ability.

5. No induction has been produced when an oral cone graft is separated from the host tissue by an agar or a cigarette paper barrier. Direct close contact between the graft and the host appears to be necessary for induction to be produced.

6. Grafts of agar on to which oral cones have previously been squashed, macerated oral cones or killed oral cones do not produce induction. If macerated oral cone tissue becomes reorganized into a small fragment possessing the histological structure of part of an oral cone, induction may then occur. The separated constituents of differentiated tissue do not appear to be capable of producing induction.

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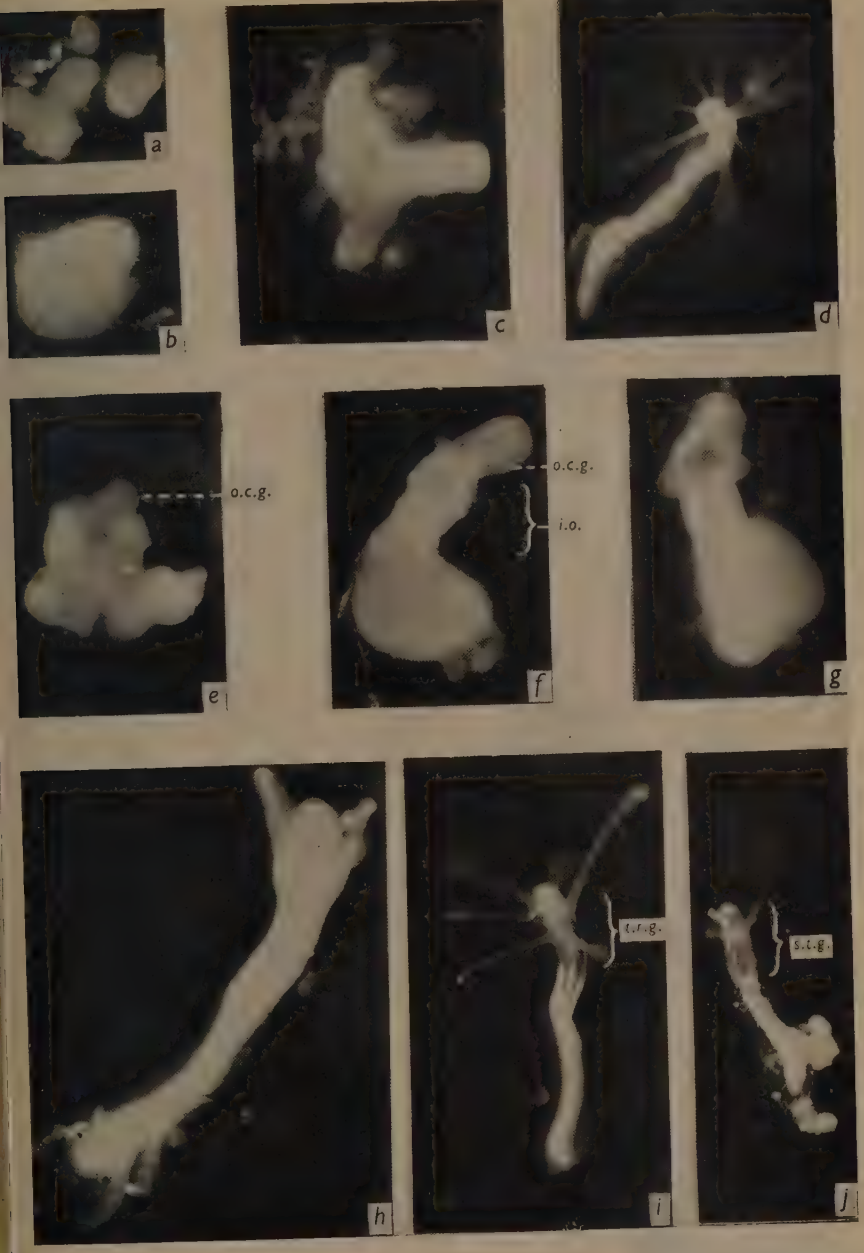

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EXPLANATION OF PLATE

(*a*)–(*d*) The stages of development of a reconstitution mass: (*a*) Mass fragments, already partly aggregated (×48). (*b*) Aggregated mass, aged 47 hr. (×48). (*c*) Outgrowths, aged 70 hr. Perisarc (more transparent) laid down around mass (×48). (*d*) Hydranth, aged 250 hr. (×20).

(*e*)–(*h*) The stages of development of a mass with an oral cone graft: (*e*) Aggregated mass with oral cone graft (*o.c.g.*), 1 hr. (×48). (*f*) Outgrowth induced (*i.o.*) by oral cone graft (*o.c.g.*), 69 hr. (×48). (*g*) Appearance of induced tentacle buds, 47 hr. (×56). (*h*) Induced hydranths, 192 hr. (×36).

(*i*) Hydranth induced by a tentacular ring graft (*t.r.g.*), showing the greater transparency of parts derived from the graft, 142 hr. (×18).

(*j*) Hydranth induced by a subtentacular region graft (*s.t.g.*), 191 hr. (×10).

The photographs were all taken from different masses, in the living condition by the late Mr F. S. Pittock, F.R.P.S. of University College, London.

THE SWIMBLADDER AND THE VERTICAL MOVEMENTS OF TELEOSTEAN FISHES

II. THE RESTRICTION TO RAPID AND SLOW MOVEMENTS

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(With Six Text-figures)

CONTENTS

INTRODUCTION

It has been shown (Jones, 1951) that a closed swimbladder may restrict the extent of rapid movements made by a teleost above its plane of equilibrium and the speed at which the fish could migrate from deep to shallow water. Experiments which were made on perch, *Perca fluviatilis* Lin., to see if their movements were subjected to these restrictions are described in the present paper.

THE RESTRICTION TO RAPID MOVEMENTS

A closed swimbladder can impose two restrictions to rapid ascents (Jones, 1951). First, it may limit the region above the plane of equilibrium within which the fish can swim freely and remain at any level, and secondly, it may restrict the excursions that the fish might make outside this zone. Observations were made on small perch subjected to reductions in pressure to determine the extent to which their movements were restricted by the presence of the swimbladder.

Methods

Two series of experiments were made, the first with fish adapted to a 0·6 m. head of water and the second with fish adapted to a 6·7 m. head of water.

(1) *First series of experiments*

The experimental tank was a 20 l. glass vessel connected to a mercury manometer and a pressure reservoir as shown in Fig. 1. The pressure in the tank was controlled

by a pin valve. The vessel was filled with water to a depth of 60 cm. and a perch kept in the tank for 24 hr. before an experiment was made. As the depth of water in the tank was equivalent to a pressure of 4 cm. Hg on the bottom, the fish was considered as being adapted to a total pressure of 76+4=80 cm. Hg. The pressure was reduced slowly in stages of 1 cm. Hg and observations made on the behaviour of the fish after each reduction.

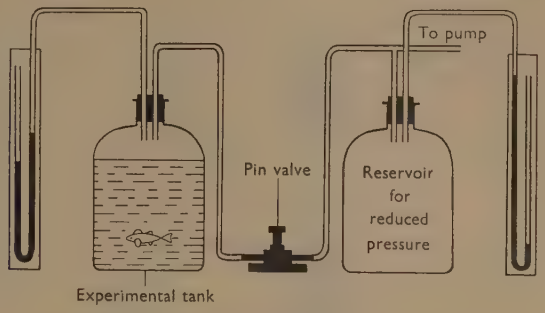

Fig. 1. Apparatus to observe the behaviour of perch adapted to 80 cm. Hg when subjected to a reduction in pressure.

(2) *Second series of experiments*

The apparatus was set up as shown in Fig. 2. Water passed from the reservoir into the experimental tank some 6 m. below. The total pressure to which the fish was subjected was equivalent to 131 cm. Hg, and to ensure complete adaptation it was kept in the tank for a week. The fish was fed during its week of imprisonment with chopped worm delivered daily through the feeding tube. Water flowed through the tank at the rate of 50 ml./min., this being controlled by the flow control which also prevented the tank from emptying if the water supply failed. After a week the flow-control clip was closed and the tap supplying the reservoir turned off. The pressure within the tank was reduced by relaxing the flow-control clip.

Results

Preliminary observations

Preliminary observations were made on perch adapted to a pressure of 80 cm. Hg. After 24 hr. in the tank they were still heavier than the water, and when swimming in midwater sank slowly to the bottom. The movement was partially checked by the pectoral fins and lateral movements of the tail. The pectoral fins beat alternately, and the effective stroke of each beat was forwards and downwards. When the pressure was reduced by 3–7 cm. Hg the fish tended to rise to the surface. The movement was checked by the pectoral fins whose effective stroke was now forwards and upwards. As the pressure was further reduced the pectorals beat faster. When the pressure was reduced by 20–30 cm. Hg the perch were very excited and had difficulty in maintaining their stations. After a 40–50 cm. Hg reduction they floated

on the surface upside down and would dart down to the bottom if disturbed, only to be carried to the surface again a few seconds later.

These preliminary observations were followed by more detailed experiments in which the time taken to make ten consecutive beats with one fin was measured with a stopwatch. Fifteen experiments were made with fish adapted to 80 cm. Hg and five with fish adapted to 131 cm. Hg. Each experiment took from 20 to 25 min.

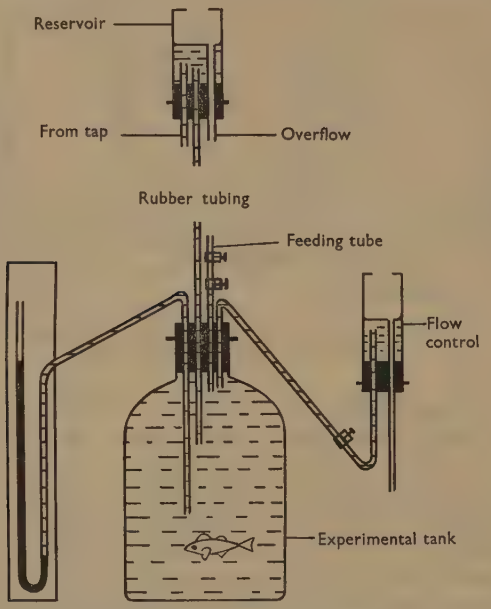

Fig. 2. Apparatus to observe the behaviour of perch adapted to 131 cm. Hg when subjected to a reduction in pressure.

The pattern of behaviour

As the pressure was reduced, the pectoral fins slowed down and then speeded up. The data is set out in Tables 1 and 2, and two typical records are shown graphically in Fig. 3.

From the experiments made on perch adapted to a pressure of 80 cm. Hg the following picture was built up of their behaviour when they were subjected to a reduction in pressure. At the beginning of an experiment a perch sometimes rested on the bottom of the tank, and no record could be made of the fin movements until the density of the fish was less than that of the water. In other cases the fish was poised in midwater and complete records were made. When the pressure was first reduced the fins beat at a slower speed. At a pressure reduction of 3–7 cm. Hg the fish no longer sank but tended to rise in the water, and the effective stroke of the fins changed to forwards and upwards. As the pressure was reduced the fins beat

Table 1. *Time in seconds for ten consecutive pectoral fin beats. Perch adapted to a pressure of* 80 *cm.* Hg

Individual fish no. ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pressure reduction in cm. Hg															
0	—	4	5	5	4·9	—	—	4·9	—	—	—	7	—	7·9	7·5
1	—	—	5	5·5	—	—	—	4	—	—	—	9	—	—	9
2	—	—	5·1	6·5	4·5	—	—	5·3	—	—	—	9·5	—	—	9
3	—	4·5	5·8	8·1	—	—	—	5	—	—	8·3	9	—	8·3	—
4	—	5	7	10·2	—	—	—	5·6	—	—	8·8	10·6	—	8·2	8·8
5	—	5·6	—	9·6	10·5	8·6	9·6	5·5	—	—	8	10·2	—	8·6	8·7
6	6·8	—	—	8·6	—	9·1	9·3	6·6	—	—	7·1	9·2	—	8·6	8·7
7	—	5·5	6·3	8	8·3	8·3	9·8	7·6	10·9	—	6·9	9·3	8·3	8·6	8·8
8	—	7·1	10·4	7·8	8·1	7·1	9·3	7·8	11·3	—	6·7	8·7	8·4	8·1	7·8
9	6	—	8·6	8·1	—	7·1	7·5	6·6	9	7·5	6·1	8·1	7·5	7	7
10	—	8·5	7·5	7·7	7	7	—	6	8·5	8	5·8	7·6	7·5	6·3	6·1
11	—	—	6·8	6·5	—	6·8	—	—	—	—	—	6·5	—	—	—
12	—	7	7·3	—	6·9	6	6·3	5·5	7·5	—	5·2	6·2	5·9	5·9	6
13	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—
14	4	—	—	—	—	4·6	—	—	—	—	5	—	—	5·8	—
15	—	6·3	5·5	5·8	5·1	4·5	5·3	4	6·3	—	4·7	5·3	5	—	5
16	—	5·8	—	—	—	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	4·5	—	—	—	—	4·1	—	—	—	—
18	—	—	—	—	—	—	—	—	5	—	—	—	4·3	5	4·6
20	3·8	4	4	4·1*	4·5*	4·8	4·8	4	4·5	—	4	4·2	4·1	*	*
22	—	—	—	—	—	—	—	—	4·5	—	*	—	—	—	—
25	—	3·5*	3·6*	—	—	*	4*	4*	*	—	—	4·5*	*	—	—
30	*	—	—	—	—	—	—	—	—	*	—	—	—	—	—

* Pressure reduction at which the compensatory swimming movements became inadequate.

Table 2. *Time in seconds for ten consecutive pectoral fin beats. Perch adapted to a pressure of* 131 *cm.* Hg

Individual fish no. ...	1	2	3	4	5	Individual fish no. ...	1	2	3	4	5
Pressure reduction in cm. Hg						Pressure reduction in cm. Hg					
0	7·9	4·5	5·2	6·7	8·5	17	—	8	6	6	6·8
1	—	4·5	5·8	9	8·6	18	5	7·7	5·8	6·3	7
2	10·2	4·5	6·2	8·6	9	19	—	7	5·5	5·3	6·7
3	—	4·8	6·5	8·1	8·3	20	5	7	5·9	6	6·5
4	7	5	7·6	8·5	8·3	22	5	5·8	5·1	6	6
5	—	5	9·3	8·5	8	24	5	5·3	5	5·3	5·5
6	7	5·1	9·1	7·7	7·8	26	5	5·1	5	5·2	5·2
7	—	5	7·6	8	8	28	4·9	5·2	4·5	4·7	5
8	6·5	5·3	7·7	8	7·7	30	5	4·8	4·3	4·5	4·5
9	—	5·8	7·2	7·5	7·7	32	5	4·6	4·6	4·5	4
10	6·1	6	6·7	7·5	7·6	34	4·8	4·7	4	4	4·1
11	—	6·4	6·8	7·3	7·5	36	4·8	4·8	4	3·6	4·2
12	5·8	7	6·3	7·2	7·6	38	—	4·7	4	3·7	4
13	—	7·3	6 5	7·1	7·7	40	—	4	4*	3·8	4
14	5·4	9·8	6·5	6·8	7·2	42	*	—	—	3·5	*
15	—	8·5	6	6·3	6·7	44	—	—	—	—	—
16	5·4	8·3	6·1	6·5	7	46	—	—	—	*	—

* Pressure reduction at which the compensatory swimming movements became inadequate.

faster. Up to a pressure reduction of about 15 cm. Hg the fish compensated in a horizontal position and remained poised a few centimetres off the bottom of the tank. It never rose for more than a few millimetres at a time and always recovered its original position. Sometimes it cruised slowly round the tank, but made no movement to suggest discomfort or alarm.

At a pressure reduction of 15 cm. Hg and over, the fish became restless and remained compensating in one position for only short periods. Between spells of compensation it moved slowly up and down the tank, circling the bottom and occasionally darting rapidly from one side to the other. The perch could still maintain its station, the pectoral fins making ten strokes in 4–5 sec.

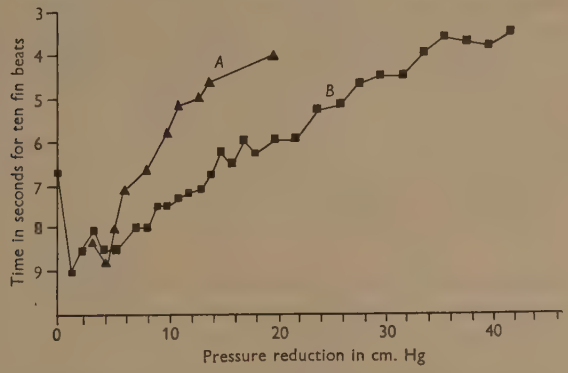

Fig. 3. Time in seconds to make ten consecutive pectoral fin beats at different pressure reductions. Record A: perch adapted to 80 cm. Hg; data from Table 1, fish 11. Record B: perch adapted to 131 cm. Hg; data from Table 2, fish 4.

The compensatory movements showed signs of being unable to resist the force tending to carry the fish to the surface when the pressure reduction was between 20 and 25 cm. Hg. When poised in midwater the fish no longer took up a horizontal position. The tail was well above the head and the body inclined to the bottom of the tank at an angle of 20°. The pectorals beat vigorously, making ten strokes in 3–4 sec. The fish was very active and excited.

At this stage the behaviour of the fish was characterized by three different movements. The first was a series of rapid leaps and darts during which the fish often hit the sides of the tank. It would rest for several seconds between these outbursts and just manage to keep its station. The second movement was a rapid circling of the tank with the nose on the bottom and the tail well above the head. After several circuits the fish would suddenly stop and pirouette around one particular spot with its nose on the bottom and body up almost vertical. The pirouette was the last of the three movements, and after gyrating round on its nose for 5–10 sec. the perch would leap away to the far side of the tank.

When the pressure was reduced by 22–30 cm. Hg the fish was no longer able to maintain its station and was carried slowly to the surface. All attempts at compen-

sation soon stopped, the fish dashing all over the tank and becoming more excited as the pressure was reduced further. At this point the experiments were concluded and the pressure restored to normal.

The fish adapted to 131 cm. Hg behaved similarly to those adapted to 80 cm. Hg, there being a proportional difference in the pressure reductions at which the pattern unfolded. The results for the two series of experiments are summarized in Table 3.

Table 3. *Summary of experimental results*

	No. of exps.	Total pressure in cm. Hg to which the fish were adapted (*a*)	Mean pressure reduction in cm. Hg at which the compensatory swimming movements became inadequate (*b*)	Standard deviation from the mean in cm. Hg	Percentage reduction in pressure ($b/a \times 100$)
Series 1	15	80	25	3	31·3
Series 2	5	131	43	2	32·8

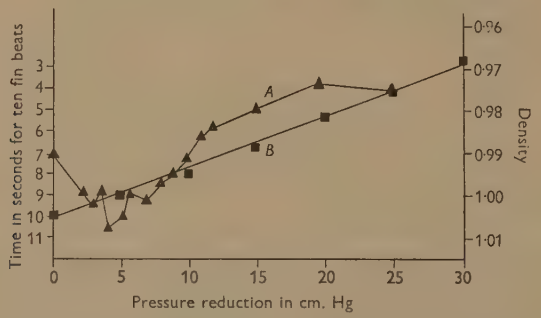

Fig. 4. Record A: time in seconds to make ten consecutive pectoral fin beats at different pressure reductions; data from Table 1, fish 12. Record B: density of perch at different pressure reductions; data from Jones (1951), Table 3.

Discussion

In Fig. 4 the time taken to make ten consecutive pectoral fin beats and the change in density of the perch (Jones, 1951) are plotted against the pressure reduction. The fins slowed down as the density of the fish approached that of the water and speeded up as the density decreased further. As judged by their slow movements up and down the tank perch adapted to a pressure of 80 cm. Hg did not appear to be very comfortable when the pressure was reduced by 15 cm. Hg and over. At a pressure reduction of 10 cm. Hg they appeared to be calm and rarely moved about the tank when they were compensating. While observations of this sort are necessarily subjective, the overall impression gained was that the perch were 'quite at home' under a pressure reduction of 10–15 cm. Hg, but this was not so when the pressure

was decreased further. The compensatory swimming movements would appear to allow a fish to maintain its station without signs of discomfort up to a pressure reduction of 10–15 cm. Hg when its density is from about 0·995 to 0·988. As the perch were adapted to a total pressure of 80 cm. Hg the percentage pressure reduction is from 13 to 19%, with a mean of 16%, a little under one-sixth. This result gives an estimate of the upper limit to the zone within which the fish can move freely and remain at any level. Its distance above the plane of equilibrium would be equal to a reduction in depth equivalent to a pressure reduction of about one-sixth of the total pressure to which the fish was adapted.

The upper limit to excursions outside this zone will depend on the force that can be resisted by the compensatory swimming movements. The results have shown that perch adapted to a pressure of 80 cm. Hg cannot maintain their stations when the pressure is reduced by 25 cm. Hg and those adapted to 131 cm. Hg when the reduction is 43 cm. Hg. The percentage pressure reductions are 31·3 and 32·8% (Table 3), and the mean of 32·03% may for convenience in calculation be approximated to one-third. At pressure reductions greater than this the perch are carried up to the surface. The upper limit to a rapid ascent is therefore equal to a reduction in depth equivalent to one-third of the total pressure to which the fish was adapted.

An example will show how these two restrictions might affect the movements of perch under natural conditions. Living at a depth of 20 m. a perch will be adapted to a pressure of 3 atm. A pressure reduction of one-sixth being equivalent to a reduction in depth of 5 m., the zone within which the fish can swim freely would therefore have its upper limit at a depth of 15 m. The fish would be mainly confined to this zone, but excursions could be made outside it. One-third of the total pressure being equivalent to a reduction in depth of 10 m., the upper limit to such movements would be the 10 m. level. Unfortunately, field data are not yet available to check the interpretation of the experimental results, and until such data have been obtained, the results must only be taken as suggesting the order of the restrictions that the presence of the swimbladder might impose to the rapid ascents of the perch.

There remains for discussion the behaviour of the perch when the pressure is reduced by 20 cm. Hg and over. They are thoroughly alarmed, and in the circling and pirouette movements appear to be trying to pass through the bottom of the tank. Under natural circumstances these 'escape movements' would take the fish into deeper and safer waters. As they occur while the fish can still maintain its station they would prevent it from making an ascent that might result in it being carried forcibly to the surface. Before the critical pressure reduction was reached the 'escape movements' would intervene and the fish seek deeper water. There would be no danger of the bladder wall rupturing because the 'escape movements' dominate the behaviour pattern when the total pressure is reduced by about one-third, and the swimbladder wall only gives way when it is reduced by three-fifths (Jones, 1951). So the rupture of the bladder wall would never be a limiting factor to a rapid movement.

A question which arises in connexion with these observations is the stimulus to which the perch are responding. The results of unpublished experiments show that

perch do not respond to the stretching of the bladder wall, and this confirms the results obtained by Franz (1938) and is in agreement with Dijkgraaf's (1942) conclusion that the bladder wall itself is not a pressure receptor. Furthermore, removal of both eyes (unpublished experiment) does not appear to upset the behaviour of the fish. This leaves the labyrinth, sense organs in the skin or the lateral line as possible sources of stimulation. Further experiments are needed to decide between them.

THE RESTRICTION TO SLOW MOVEMENTS

The speed at which a physoclist can pass from one level to another in the course of diurnal, seasonal or other periodic migrations will depend on how quickly it can adjust the volume of the swimbladder to the pressure change involved in the movement (Jones, 1951). Brown (1939) found that the guppy, *Lebistes reticulatus*, became adapted to a reduction of 10 cm. Hg in about 2 hr., but little else is known about the speed with which a physoclist can restore its hydrostatic equilibrium when subjected to a reduction in pressure. Experiments were therefore made on perch to see how long they took to adapt themselves to different pressure reductions.

Methods

A fish was subjected to a known pressure reduction and the time taken for adaptation recorded. When lighter than the water the fish made compensatory movements to maintain its station, but when adaptation was complete it would rest either on the bottom or in midwater without moving. Two series of experiments were made. In the first, perch were subjected to a pressure reduction under laboratory conditions. In the second, perch were trapped at known depths in Lake Windermere, taken into the laboratory, and the time that they took to become adapted to the change of pressure recorded.

(1) *First series of experiments*

In these experiments the fish were adapted to a total pressure of 80 cm. Hg. The experimental tank was filled with water and connected to a manometer and a length of rubber tubing as shown in Fig. 5. To reduce the pressure the rubber tube was lowered until a reduction of 10 cm. Hg was registered by the manometer, giving a percentage reduction of 12·5. Water running over the tank kept the temperature constant to $\pm 0{\cdot}5^{\circ}$C. Twenty-three experiments were made, and each fish observed almost continuously throughout an experiment. During a long experiment a perch would occasionally come up to the top of the tank and rest against the glass without making any compensatory movements. As Meesters & Nagel (1934) showed that gas resorption would then slow down and cease, the tank was disturbed and the perch would dart down to the bottom and continue compensating.

(2) *Second series of experiments*

Perch were trapped in Windermere at depths of 3 m. (10 ft.) and 4·6 m. (15 ft.). The traps were lifted every 24 hr. and the fish assumed to be adapted to the pressure at the depth from which they were taken. Perch trapped at 3 m. were subjected to a 25% reduction in pressure when brought to the surface, and those taken from

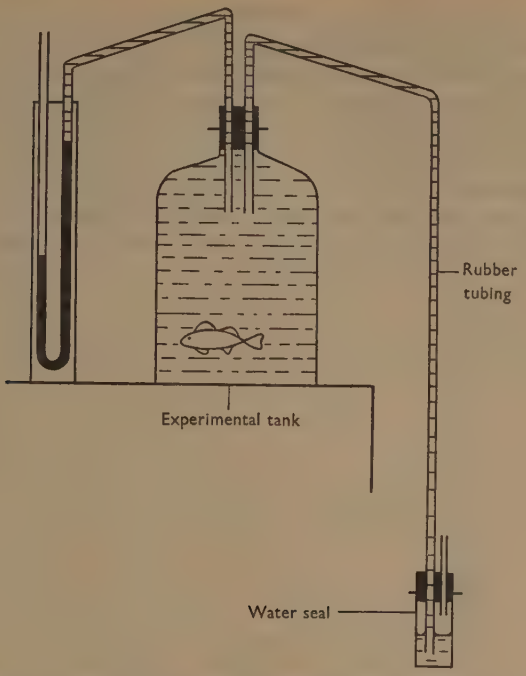

Fig. 5. Apparatus to observe the time taken by perch adapted to 80 cm. Hg to recover their hydrostatic equilibrium when subjected to a pressure reduction of 10 cm. Hg.

Table 4. *Time in hours for perch to become adapted to different pressure reductions*

Percentage reduction of the total pressure to which the fish was originally adapted	No. of exps.	Time in hours for adaptation $\bar{x}$	Standard deviation in hours s	Coefficient of variation ($s/\bar{x} \times 100$)
12·5	23	2·5	1	40
25·0	72	9·0	3	33
33·3	75	16·0	4	25

4·6 m. to a 33% reduction. Running females and injured fish were discarded and the remainder taken into the laboratory and each fish placed in a separate glass vessel of about 15 l. capacity. The time taken for adaptation was recorded. Seventy-two fish were taken from 3 m. and seventy-five from 4·6 m.

Results

The results are summarized in Table 4 and shown graphically in Fig. 6.

Discussion

Some fish adapted themselves quicker than others. This may reflect the variations in the percentage volume of the swimbladder (Jones, 1951) or differences in the composition of the swimbladder gas. Meesters & Nagel (1934) and Brown (1939) have shown that adaptation proceeds most rapidly and effectively when the initial concentration of CO_2 is high. Furthermore, not all the perch in the traps might have been adapted to the depth at which they were caught. They could have come from deeper or shallower water and entered the traps at any time during the 24 hr. they were down. So a certain amount of variation was to be expected. Although the coefficient of variation (Table 4) is greater in the laboratory than the field

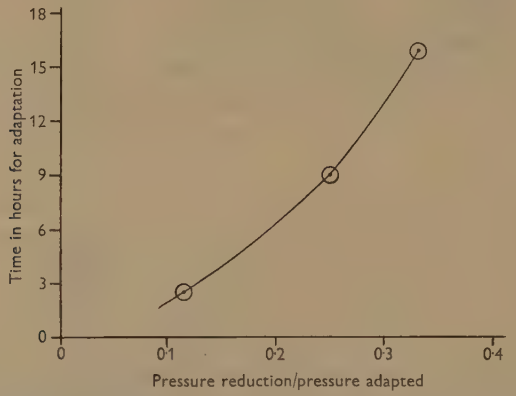

Fig. 6. Time in hours taken by perch to recover their hydrostatic equilibrium when subjected to different pressure reductions. Data from Table 4.

experiments, there is no reason to believe that the difference is significant, and in view of the number of fish used the results are considered to be reliable. The graph in Fig. 6 has not been extrapolated to the origin, as the results of Meesters & Nagel (1934) show that adaptation to a very small pressure reduction might proceed very quickly due to the initial escape of CO_2 from the bladder.

It has been shown (Jones, 1951) that the quickest way in which a fish could migrate from deep to shallow water would be to swim up rapidly to the highest level at which it could maintain its station satisfactorily and then to proceed on slowly adapting the volume of its swimbladder to avoid any further decrease in density. The minimum time taken for a fish to swim from a depth of pressure P_1 to shallower water of pressure P_2 can be calculated from the equation

$$T = B \log_e \frac{P_1}{P_2} - C, \qquad (12)$$

where T is the time in hours, B a factor for the rate of adaptation and C a correction for the initial rapid ascent (Jones, 1951).

The zone within which a perch can swim freely has been shown to have its upper limit at a distance above the plane of equilibrium equivalent to a reduction of one-sixth of the total pressure to which the fish is adapted. If it is assumed that the fish swims up to this level rapidly at the beginning of the migration then

$$C = \log_e \tfrac{6}{5} = 0{\cdot}1823 \quad \text{(Jones, 1951)}.$$

Having arrived at this level the fish swims on slowly. The value of B can be found from the graph in Fig. 6 and will be equal to the gradient of the curve at a pressure reduction of one-sixth:

$$B = \frac{T}{dp/P} = \frac{4}{\frac{1}{6}} = 24.$$

Allowing for a standard deviation of 1·5 hr. in the time for adaptation B might be expected to have values between $2{\cdot}5/\frac{1}{6} = 15$ and $5{\cdot}5/\frac{1}{6} = 33$. Substituting for B and C in equation (12)

$$T = 24 \log_e \frac{P_1}{P_2} - 0{\cdot}1823.$$

Table 5. *The minimum time in which a perch would be expected to make vertical movements involving reductions of* 10–90% *in the total pressure to which it was originally adapted*

Percentage reduction of the total pressure	Mean time in hours $B=24$	Expected variation in hours	
		$B=15$	$B=33$
10	—	—	—
20	1	0·5	1·5
30	3·5	1·5	5
40	8	5	11
50	12	7·5	17
60	17·5	11	24
70	24	15	33
80	34	21	46
90	50	32	70

The minimum time in which a perch could move from deep to shallow water can now be calculated, and Table 5 gives the time in which a perch would be expected to make vertical movements involving pressure reductions of 10–90% of the total pressure to which the fish was originally adapted. From these results it is possible to estimate the extent to which the presence of the swimbladder might restrict the vertical movements of perch. Diurnal migrations usually begin around sunset and are complete by midnight at the latest. So at the maximum 5–7 hr. would be spent over the ascent. Reference to Table 5 shows that perch would not therefore be expected to make diurnal migrations involving pressure reductions of more than 40% of the total pressure to which they were adapted. And it follows that if a perch is to reach the surface around midnight it would have to be adapted to a depth of not more than 6–7 m. during the day.

While nothing is known about the diurnal migrations of *Perca fluviatilis*, studies have been made on the related American yellow perch, *P. flavescens*. Hasler

& Bardach (1949) have shown that the yellow perch migrates inshore from deep to shallow water, moving from a depth of about 13 to 6 m. in 2–3 hr. (Bardach, 1949). The movement involves the total pressure to which the fish were originally adapted being reduced by 31%, and the time taken falls within the expected range of 1·5–5 hr. But more field observations are required to confirm the experimental results.

GENERAL DISCUSSION

From the results of the experiments made on perch it would appear that these fish can only swim freely above their plane of equilibrium within a narrow zone whose height is equivalent to a 16% (one-sixth) reduction in the total pressure to which they are adapted. Excursions from this zone would not involve more than a 33% reduction of the total pressure. The results suggest that perch would not make diurnal migrations that involved a 40% reduction in pressure, and that the minimum time in which they could swim from deep to shallow water would be of the order given in Table 5.

Although the interpretations of the experimental results are not yet confirmed by field observations, the discussion may be extended, with caution, to marine physoclists. As the swimbladder is relatively smaller in marine than in fresh-water teleosts (Jones, 1951), a greater pressure reduction will be required to produce a given density decrease in the former than in the latter. When perch adapted to a pressure of 80 cm. Hg are subjected to a reduction of 10–15 cm. Hg, their sinking factors decrease from 1005 to 995–988 (Fig. 4). The same decrease occurs when the fish swims to the top of the zone within which it can swim freely. But to produce a similar decrease in the sinking factor of the wrasse, *Crenilabrus melops*, the pressure must be reduced by 15–20 cm. Hg (Jones, 1951). Other things being equal, the zone within which the wrasse could swim freely would therefore extend above its plane of equilibrium for a distance equivalent to a 22% reduction in pressure as compared with 16% for the perch. If the movements of physoclists were restricted in this way they would be expected to keep to a fairly steady depth when swimming in midwater. Sund (1935) found this to be true of spawning cod in the Loften area. Echo sounding showed the fish concentrations to be limited to a sharply defined layer 10–12 m. thick which was widespread at a depth of 72 or 50 m. The bottom of the layer would be at a depth of 84 or 62 m. If the cod were adapted to these depths a rapid ascent of 12 m. to reach the top of the layer would involve a 13 or 17% reduction in the total pressure. Such a movement is consistent with the interpretation of the experimental results.

As the speed with which marine physoclists become adjusted to pressure reductions is not known, any estimate of the restriction that the swimbladder might make to their diurnal migrations is largely guesswork. But assuming that they can adapt themselves at the same speed as perch, and making a correction for C in equation (12) as the zone within which they could swim freely would be greater than that of a fresh-water teleost, it can be shown that they would not be expected to make a diurnal migration that involved a reduction of more than 50% in the total pressure

to which they were originally adapted. Fish adapted to the pressure at the sea-bottom by day would not therefore be expected to appear in the upper waters by night. Hake spend the day on the bottom which they are believed to leave at night to follow the euphausids on which they feed (Hickling, 1925, 1927). But how far they swim up is not known.

The experimental results are of interest in connexion with the deep scattering layer. The layer is the reflexion of the outgoing signal of an echo sounder which appears as a false bottom at various depths in the Pacific and Atlantic oceans. It shows an extensive migration from a depth of 300–700 m. by day up to a depth of 200 m. to the surface by night and descends again in the morning. Each movement is complete in about an hour and a half. The diurnal cycle suggests that the phenomena is caused by migrating organisms rather than by a physical discontinuity, and it has been suggested that the scatterers are zooplankton, fish or squid. At present zooplankton, and euphausids in particular, are favoured as the scatterers. (Dietz, 1948; Hersey & Moore, 1948; Johnson, 1948; Moore, 1950; Boden, 1950). Chapman (1947) has suggested fish as being responsible for the phenomena, and Johnson (1948) believes that the rate at which the layer ascends or descends may be an aid in deducing what animal groups take part in the movement.

The pressure change involved in the migration is from about 50 to 10 atm. or less, a reduction of at least 80%. From Table 5 it will be seen that a perch would be expected to take about 34 hr. over such a movement, so it does seem unlikely that a teleost with a closed swimbladder could complete the ascent in 1–2 hr. But if the percentage volume of the swimbladder was reduced with a corresponding increase in the fat content of the tissues (Taylor, 1921), or if the bladder wall was thickened to resist the expansion of the bladder gas, or the rate of gas resorption was 20–30 times quicker than has been supposed, a physoclistous fish might be able to make such an extensive and rapid diurnal migration. If fish with closed swimbladders are partly responsible for the deep scattering layer they would be expected to show one or other of these adaptive features.

While physoclists can only remove gas from the swimbladder by resorption, physostomes, in which the connexion between the bladder and the alimentary canal is retained, can get rid of it quickly through the pneumatic duct. Physostomes, such as the herring and the pilchard, are known to move rapidly from deep to shallow water. Using an echo sounder Hodgson & Richardson (1949) followed the vertical movements of a pilchard shoal in response to changes of light intensity with results that show that these fish can accommodate themselves rapidly to reductions in pressure. The shoal moved from a depth of 15 to 7 m. in 3 min. Burd & Lee (1951) have recorded diurnally migrating layers in the English Channel and Arctic waters which are probably caused by young herring and young pilchards.

Many primitive teleosts, such as the Clupeiformes, Anguilliformes, Cypriniformes and Mormyriformes, retain the pneumatic duct when the bladder is present, but the duct is lost and the swimbladder closed in most of the higher teleosts, such as the Perciformes, Beryciformes, Thunniformes, Gadiformes and Lophiiformes. This raises the question as to whether there is a functional significance in the loss of

the pneumatic duct. The closed swimbladder may be concerned with gas conservation, as physostomes, with poorly developed red bodies (Woodland, 1911), can only replace that lost through the duct by gulping air at the surface or by a very slow process of secretion (Ledebur, 1937). Furthermore, a closed swimbladder might enable a fish to keep at a steady depth when other factors, such as light intensity, are of little help. On the other hand, physostomes might have an advantage over physoclists in a prey-predator relationship, such as herring escaping from cod or pike in pursuit of perch. The loss of the swimbladder, as in the mackerel, *Scomber scombrus*, would allow a fish to move freely from one level to another.

Although the experiments described in this paper show that the swimbladder will restrict the vertical movements of a physoclist so that the habits of these fish must differ from those in which the bladder remains open or is lost in the adult, the swimbladder serves such a variety of functions that it is probably more of an asset than a liability even when it is closed. For example, Plattner (1941) has shown that it has important hydrostatic functions, it is used in sound production (Knudsen, Alford & Emling, 1948), and preliminary experiments by Griffin (1950) have shown that when coupled to the ear it may probably increase the sensitivity of hearing twenty-fold.

SUMMARY

1. Perch can only swim freely above their plane of equilibrium within a narrow zone whose height is equivalent to a 16% (one-sixth) reduction in the total pressure to which they are adapted. Excursions from this zone would not involve more than a 33% reduction of the total pressure.

2. Calculations were made of the minimum time in which perch could make slow vertical movements involving reductions of 10–90% in the total pressure to which they were originally adapted, and it was concluded that perch would not make diurnal migrations in which the reduction was more than 40% of the total pressure.

3. It is unlikely that teleosts with closed swimbladders would be able to make diurnal migrations as extensive and rapid as that shown by the deep scattering layer unless the swimbladder was reduced in size, the bladder wall thickened to resist expansion of the gas, or the rate of gas resorption 20–30 times quicker than in the perch.

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THE EXCRETION OF SODIUM AND POTASSIUM BY THE MALPIGHIAN TUBULES OF *RHODNIUS*

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(*Received* 18 *June* 1951)

(With Four Text-figures)

I. INTRODUCTION

About twenty years ago a very full account of the physiology of excretion in the blood-sucking insect *Rhodnius prolixus* Stål was given by Wigglesworth in three papers (Wigglesworth, 1931 *a*, *b* and *c*), and in order to place in proper perspective the problems which are the concern of the present investigation it is necessary to give a brief survey of the position as Wigglesworth left it.

First, in his investigation of the chemical composition of the urine he showed (among other things) that most of the fluid of the blood meal was excreted within 3 or 4 hr. as a clear urine, mainly a solution of sodium and potassium chlorides more or less isotonic with the ingested blood. In the first drops of urine passed after feeding the proportion of sodium to potassium was high, but during the next 2 or 3 hr. the proportion of sodium to potassium fell steadily until at the end of this 'diuresis period' the relative proportions were reversed. Thereafter the urine was generally retained in the rectum; it developed a yellow colour, its osmotic pressure increased and a white sediment of 'uratic spheres' accumulated.

Secondly, Wigglesworth described the whole system of excretory organs. There are four Malpighian tubules each about 4·35 cm. long. The upper or distal portion of each tubule, constituting about two-thirds of its whole length, is translucent and colourless, or faintly yellow; the striated inner border is of the honeycomb ('wabensaum') type. The cells of the lower or proximal portion are almost transparent, and the striated border is of the brush ('bürstensaum') type. Each Malpighian tubule enters the rectum through a small ampulla, the four ampullae being grouped around the opening of the midgut. The ampullae contain very large cells with long mobile processes which may extend into the rectum. The rectum is a muscular sac capable of considerable distension, and the glandular region of the epithelium is confined to the area around the opening of the midgut and Malpighian tubules.

Thirdly, he investigated the process of excretion, showing that fluid was secreted into the upper portion of the tubule and reabsorbed from the lower portion with the deposition therein of uratic spheres. He showed that the contents of the upper portion were weakly alkaline, those of the lower portion distinctly acid. These observations he assimilated to the following theory of the excretory mechanism. A solution of sodium and potassium urates is secreted into the upper portion of the

tubule; on passing down to the lower portion it is made acid with CO_2; this causes precipitation of uric acid as uratic spheres; sodium and potassium are reabsorbed as a solution of bicarbonates.

That these interesting observations have remained so long without being followed up seems probably due to the inadequacy of standard chemical methods for dealing with the small quantitities of fluid available. But the position has improved in recent years with the development of spectrometric and activation techniques; it has now become possible to determine sodium and potassium by flame photometry and osmotic pressure by freezing-point depression on samples of the order of one-thousandth of a cubic millimetre. The present paper gives an account of the application of these techniques to two of the questions which arise from the original work of Wigglesworth, namely,

(i) whether the variations of sodium/potassium ratio in the urine are associated with corresponding variations in the insect's haemolymph,

(ii) whether the histologically distinct portions of the Malpighian tubule have physiologically distinct roles in the excretion of sodium and potassium.

II. MATERIAL AND METHODS

The stock of *Rhodnius* was reared in the laboratory according to the methods of Buxton (1930). For the experiments adult insects which had been without food for at least 3 weeks were used.

The insects were usually allowed to gorge themselves to repletion on rabbit's blood. The great distension of the stomach in a fully gorged insect crowds the other organs together making dissection difficult, and for this reason in some experiments involving operations upon the Malpighian tubules the insect was removed from the rabbit at the stage when the connexivum was just beginning to unfold. In other experiments the insects were allowed to feed through a piece of mouse skin upon ox blood with varying amounts of potassium chloride added. I am very grateful to Dr Wigglesworth for drawing my attention to this method of feeding which he has himself used with *Rhodnius* but never published.

For purposes of collecting the urine a piece of brass wire was fastened with soft wax to the bases of the forewings and the insect was held over a varnished watch-glass containing liquid paraffin. If samples of haemolymph were to be taken during the course of excretion the posterior portions of the wings were cut off and a thin layer of soft wax was applied to the abdominal tergites with a heated wire. With a fine needle a puncture was made between two tergites near the lateral margin and the drop of haemolymph exuded (about 0·25 cu.mm.) was collected in a pipette, the wound being then sealed over with wax. During the later stages of excretion collections of urine were made directly from the rectum by inserting a cannula through the anus. In some cases a wide cannula was permanently inserted and the urine removed with a pipette as it accumulated.

Operations upon the Malpighian tubules were carried out under a medium power binocular microscope, the preparation being illuminated through a glass rod light-guide. The insect was fastened with soft wax to a small table, about 2 by 0·5 cm.,

mounted on a ball-and-socket joint. The posterior abdominal tergites were completely removed. It was necessary to take precautions to avoid desiccation of the preparation during these operations which often took an hour for completion. Moist chamber methods have certain disadvantages; if the chamber is small, access to the preparation is difficult, if it is large it is difficult to ensure that the air is kept fully saturated. The method used in the present investigation was as follows. A stream of compressed air was bubbled through hot water and then passed through about 10 ft. of lead tubing laid out over the bench. The air stream left the tube saturated with water at room temperature and was then directed over the preparation through a cowl. Samples of haemolymph taken at the beginning and end of the operation showed practically no increase in osmotic pressure (see Table 5, serials 14, 15 and 16).

Wigglesworth (1931*b*) has described how one tubule on each side has a forwardly directed loop in the course of which the change from upper to lower portion of the tubule is situated. In the present work the forwardly directed loop on the right-hand side was used, and in preliminary experiments in which liquid paraffin stained with Sudan III was injected it was ascertained that this loop connected with the more ventral of the two ampullae on the right-hand side. The region of the ampullae is concealed by coils of tubules, and it was first necessary to cut through the fine tracheae which connect the tubules to the rectum. When this was done the rectum could be drawn to the left and the ampullae with their entering tubules exposed. A sample was first collected from the tubule entering the ventral ampulla, representing the lower portion, and a further sample was then taken from the upper portion at its junction with the lower portion in the forwardly directed loop. In this way the composition of the fluid in the upper and lower portions could be compared on the same tubule with its tracheal supply intact.

Collections were made by grappling the tubule with a fine tungsten hook and thrusting a silica pipette into it where it passed over the hook. Both the hook and the pipette were mounted on a simple micromanipulator with independent movements. The pipette was drawn with a steep taper and was about 20 μ in diameter at the tip; it was partly filled with liquid paraffin and this was kept under slight pressure to prevent fluid entering until penetration had been effected. Unfortunately, it cannot be said that this method worked well. When urine flow was vigorous and the tubule was well distended attempts were generally successful, but the resistance of the tubule to penetration was such that thrusting with the pipette often resulted merely in dragging the tubule over the hook. In some cases penetration was only achieved by tearing the tubule and slipping it over the end of the pipette with forceps held in the hand, the hook then serving merely to prevent it sliding off. Once the tip of the pipette was seen to have entered the tubule a droplet of liquid paraffin was injected and the tip was then thrust farther in beyond the droplet. Slight suction was often necessary to start the flow of fluid into the pipette. Collection took 5–20 min. according to the rate of urine flow, and the samples taken were 0·01–0·1 cu.mm. in volume. Special attention was given to the possibility of haemolymph being drawn into the pipette through the wound in the tubule. The

pipette was always thrust in as far as possible so that its increasing diameter at the point of penetration filled the lumen of the tubule. The pipette being slightly inclined to the horizontal, it was possible to raise it so that the point of penetration was exposed while the part of the tubule in which the tip lay remained below the surface of the haemolymph. In some experiments a crystal of aniline blue was placed upon the region of penetration, but no dye could be detected in the sample. When collection was completed the pipette was raised above the surface of the haemolymph before being withdrawn from the tubule, and the sample was ejected into a watch-glass filled with liquid paraffin.

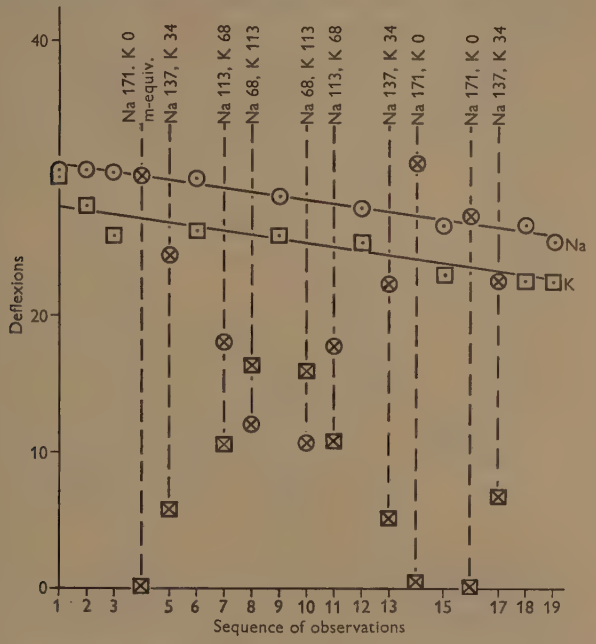

Fig. 1. A series of observations taken during the calibration of the flame photometer. The standard solution contained 171 mM. NaCl plus 171 mM. KCl/l. The concentrations of sodium and potassium in the calibrating solutions are given beside the observed deflexions. The symbols used are: for the standard solution Na ⊙, K ⊡; for the calibrating solutions Na ⊗, K ⊠. For further explanation see text.

A method has been described (Ramsay, 1950; Ramsay, Falloon & Machin, 1951) for the determination of sodium in small volumes of fluid by flame photometry. This method has now been extended to deal with potassium as well as sodium in the same sample; it is still under development, and it is hoped that a full description of it will be published in due course. Its accuracy is still rather poor, but in view of the very large variations in the concentrations of sodium and potassium indicated in Wigglesworth's studies it was felt that the method, even in its present stage, could

be usefully applied to these problems. One of the first difficulties is that throughout the course of a series of measurements the sensitivity of the apparatus varies. (This is believed to be due to the effect of temperature changes upon the refractive index of the prism.) It is therefore necessary to make determinations upon a standard alternately with determinations upon an unknown. Some of the measurements made for purposes of calibration are reproduced in Fig. 1. The volume of fluid delivered by the burette was approximately $0{\cdot}7 \times 10^{-3}$ cu.mm. The standard used for most of this work was a solution containing 171 mM. NaCl plus 171 mM. KCl/litre. Determinations were made upon the standard and upon the calibrating solutions in the order shown, and the time taken for each determination was roughly the same,

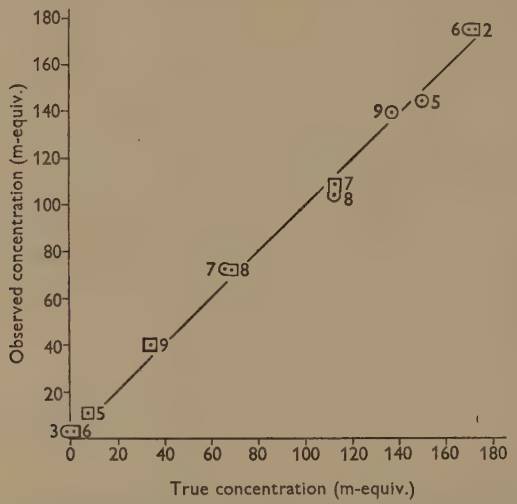

Fig. 2. The results of calibration tests with known solutions, showing the errors involved in the assumption of a linear relationship between deflexion and concentration. The symbols used are: Na ⊙, K ⊡. The figure associated with each point on the graph gives the number of observations upon which the point is based. For further explanation see text.

so that the abscissa of Fig. 1 represents a time scale. The deflexions were plotted and smooth curves were fitted by eye to the deflexions of the standard, one for sodium and one for potassium. The deflexions given by the calibrating solutions were then compared with the appropriate portions of the standard curves. To take an example. The deflexions of observation 7 are Na 18·1, K 10·9; the corresponding standard deflexions from the curves are Na 29·3, K 26·3. Assuming that deflexion is directly proportional to concentration (see below) the observed concentrations of the calibrating solution are: Na $171 \times \frac{18{\cdot}1}{29{\cdot}3} = 106$ m.equiv., K $171 \times \frac{10{\cdot}9}{26{\cdot}3} = 71$ m.equiv. The true concentrations were Na 113 m.equiv., K 68 m.equiv. so that the errors are Na -7 m.equiv., K $+3$ m.equiv. The average values of the observed concentrations are plotted against the true concentrations in Fig. 2, from which it emerges that

the error involved in the assumption of linearity is nowhere greater than 7·5 m.equiv. The scatter of the standard deflexions about the standard curves has been worked out for several series (forty-six observations in all for each element) and has an average value corresponding to ±5 m.equiv. for both sodium and potassium. A single observation might be in error by (conventionally) three times the average random error plus the systematic error, i.e. by 22·5 m.equiv. In practice two determinations were made on each unknown, and if the two determinations differed by more than 20 m.equiv.—which was very seldom—two more determinations were made. It is difficult to define the accuracy of the method in simple statistical terms, but it is reasonable to suppose that in the results here presented a difference in concentration greater than 15 m.equiv. is real and not due to the errors of analysis.

In some experiments the total osmotic pressure was determined by freezing-point depression (Ramsay, 1949). Osmotic pressure is expressed in terms of the equivalent concentration of monovalent electrolyte (e.g. NaCl) in mM./litre. These measurements were made in duplicate and the probable error is of the order of ±2mM.

III. RESULTS

(*a*) *Haemolymph of fasting* Rhodnius

The experiments on fed insects shortly to be described indicated that the concentration of potassium in the haemolymph was only about $\frac{1}{10}-\frac{1}{20}$ of the concentration of sodium. Determinations were subsequently made on the haemolymph of fasting insects with the apparatus adjusted to give maximum sensitivity to potassium and with a standard solution containing 150 mM. NaCl plus 7 mM. KCl, fairly closely approximating to the haemolymph in composition. Three insects were taken and the averages of four determinations upon each insect are given in Table 1, together with the averages of two determinations of freezing-point depression.

Table 1. *Haemolymph of fasting* Rhodnius

Serial	Na	K	O.P.
20	191	22	238
21	162	11	207
22	157	9	221

In the case of serial 20 there was so little haemolymph in the body that it was not possible to collect more than a few hundredths of a cu.mm., and this insect may have been in a state of partial desiccation. But it is clear from these measurements that the composition of the haemolymph just before feeding is not widely different from its composition just after feeding on normal blood.

(*b*) *Haemolymph and urine of naturally fed* Rhodnius

All the data under this head are assembled in Table 2, and the figures for serial 5 are plotted in Fig. 3. These results confirm the findings of Wigglesworth in that (i) the potassium concentration rises during the course of excretion, and (ii) the

occurrence of sediment and of the yellow colour are associated with a considerable rise in osmotic pressure. They also show clearly that while these extensive changes in the sodium/potassium ratio of the urine are taking place the changes in the sodium/potassium ratio of the haemolymph are relatively small. This settles the first of our questions; the Malpighian tubules and rectum are able to keep the composition of the haemolymph constant within fairly narrow limits.

(*c*) *Haemolymph and urine of artificially fed* Rhodnius

The figures in Table 2 indicate that there is a decrease in sodium concentration and an increase in potassium concentration in the haemolymph during the course of

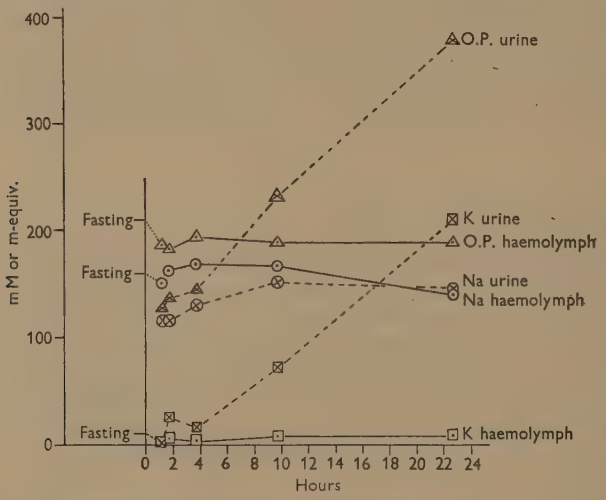

Fig. 3. For explanation see text. The 'fasting' values are based on the data in Table 1.

excretion, but the changes are small. It is therefore of some interest to ascertain what happens when the excretory mechanism is submitted to greater stress, and this was easily done by allowing the insect to feed upon blood in which the concentration of potassium had been artificially increased. The results of these experiments are shown in Table 3. The first experiments with artificial blood mixtures were carried out with approximately 3 parts of ox blood to 1 part of 171 mM. KCl. The insects fed on this quite readily and showed no ill effects, in spite of the fact that in one case (serial 3) there was appreciable increase in the potassium concentration of the haemolymph. A mixture of 1 part of ox blood to 3 parts of 171 mM. KCl was then tried, and since the method of mounting the insect for collection of urine made it difficult to observe its behaviour, some insects were fed but not mounted. Serials 17 and 18 stopped feeding before becoming fully distended and did not pass any urine, although there was a considerable accumulation of clear fluid in the rectum. The potassium concentration in the haemolymph was raised

Table 2. *Haemolymph and urine of naturally fed* Rhodnius

Serial	Temp. °C	Time after feeding	Haemolymph			Urine			Appearance of urine	How obtained
			Na	K	O.P.	Na	K	O.P.		
1	24	(*a*) 0·30	—	—	—	126	22	—	Colourless, no sediment	Passed into watch-glass
		(*b*) 2·00	—	—	—	129	25	—	Colourless, no sediment	Passed into watch-glass
		(*c*) 19·00	175	5	—	169	58	—	Colourless, some sediment	By cannulation
2	24	(*a*) 0·20	147	4	—	140	16	—	Colourless, no sediment	Passed into watch-glass
		(*b*) 0·45	180	6	—	132	10	—	Colourless, no sediment	Passed into watch-glass
		(*c*) 1·35	162	8	—	152	7	—	Colourless, no sediment	Passed into watch-glass
5	18–20	(*a*) 1·20	151	1	187	116	1	127	Colourless, no sediment	Permanently cannulated
		(*b*) 1·50	162	5	182	116	25	137*	Colourless, no sediment	Permanently cannulated
		(*c*) 3·45	169	3	194	131	16	145*	Colourless, no sediment	Permanently cannulated
		(*d*) 9·45	167	7	189	152	72	232	Colourless, some sediment	Permanently cannulated
		(*e*) 22·45	141	8	189	146	210	379	Yellow, much sediment	Permanently cannulated
6	18–20	(*a*) 0·30	156	5	180	101	8	114	Colourless, no sediment	Permanently cannulated
		(*b*) 21·30	149	6	180	220	163	392	Yellow, much sediment	Permanently cannulated
7	18–20	24·00	173	4	209	101	256	428	Yellow, much sediment	Passed into watch-glass
9	18–20	29·00	167	4	202	116	200	302*	Yellowish, some sediment	Passed into watch-glass
10	18–20	44·00	164	7	209	192	137	362	Yellow, much sediment	By cannulation
11	18–20	17·00	163	9	201	100	53	154	Colourless, little sediment	By cannulation

* See text, p. 125, l. 26.

Table 3. *Haemolymph and urine of artificially fed* Rhodnius

Serial	Temp. °C.	Mixture of blood:KCl	Time after feeding	Haemolymph			Urine			Appearance of urine	Behaviour
				Na	K	O.P.	Na	K	O.P.		
3	24	3:1	(*a*) 1·35	162	16	—	99	89	—	Colourless, no sediment	Normal response
			(*b*) 8·00	150	33	—	96	115	—	Colourless, little sediment	Normal response
4	18–20	3:1	(*a*) 1·00	152	9	—	101	74	—	Colourless, no sediment	Normal response
			(*b*) 2·30	154	5	—	97	74	—	Colourless, no sediment	Normal response
			(*c*) 24·00	155	9	—	57	137	—	Yellowish, some sediment	Normal response
17	18–20	1:3	(*a*) 0·35	121	69	—	—	—	—	—	Normal response
			(*b*) 4·40	114	96	—	—	—	—	—	Normal response
18	18–20	1:3	(*a*) 0·45	98	92	—	—	—	—	—	Normal response
			(*b*) 4·30	87	124	—	—	—	—	—	Normal response
			(*c*) 10·30	87	95	—	—	—	—	—	No response
19	18–20	1:3	(*a*) 0·25	144	13	—	112	49	—	Colourless, no sediment	Normal response
			(*b*) 1·00	146	12	—	116	59	—	Colourless, no sediment	Normal response
			(*c*) 4·00	149	14	—	99	62	—	Colourless, no sediment	Normal response
			(*d*) 10·00	142	15	—	56	161	—	Colourless, some sediment	Normal response
			(*e*) 22·00	140	15	—	22	232	—	Yellow, much sediment	Normal response
			(*f*) 30·00	116	86	—	60	143	—	Colourless, some sediment	Weak response
			(*g*) 54·00	129	41	—	60	142	—	Colourless, some sediment	No response, heart beating
			(*h*) 70·00	126	40	—	53	153	—	Colourless, some sediment	No response, heart beating

some 10 times, yet the insects continued for many hours to respond with apparently normal questing movements of the legs when their antennae were touched. Later, however, the response weakened and failed. The following observations were made on another insect which had taken a full meal but which had not been mounted for the collection of urine.

Time after feeding hr.	min.	Behaviour
0	30	Thrown upon back, succeeded in righting itself.
1	15	Thrown upon back, tried to right itself but failed.
2	45	Thrown upon back, did not try to right itself; in normal posture attempted to walk with the front pair of legs only.
3	45	Relaxed its grip on a vertical paper surface and lay motionless. No recovery.

More interest attaches to the results of serial 19, shown plotted in Fig. 4. In this case the insect was fully distended after its meal. Urine flow began almost at once and continued at a brisk rate for 4 hr. During this period the concentration of potassium in the urine was relatively high but never exceeded the concentration of sodium. The last drop of urine, passed sometime between 4 and 10 hr., was colour-

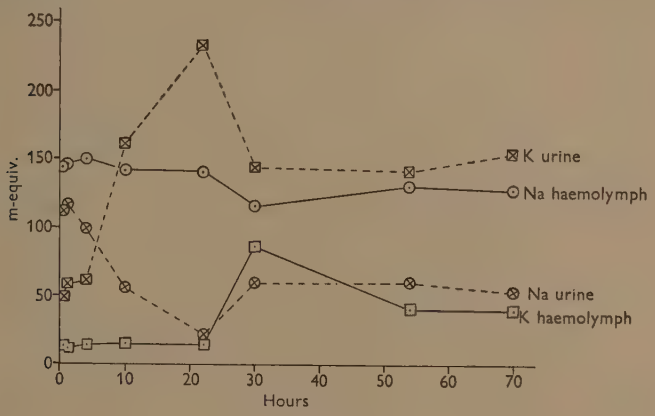

Fig. 4. For explanation see text.

less with a little sediment, and the concentration of potassium was now greater than that of sodium. At 22 hr. the rectum was cannulated, and the urine obtained was yellow with much sediment and a concentration of potassium about 10 times that of sodium. These changes in the urine normally indicate the end of the diuresis period, and up to this time the concentration of potassium in the haemolymph was still only 50% above normal and the insect was fully responsive. But sometime between 22 and 30 hr. events took a different course. The urine collected in the cannula was colourless with a moderate amount of sediment and a higher concentration of potassium than sodium, but the concentration of potassium in the haemolymph had risen to about 8 times normal and the insect showed very little response to stimulation. At 54 hr. the potassium in the haemolymph had fallen to

about 4 times normal, but the insect showed no response to stimulation although its heart could be seen beating and was still beating rather irregularly at 70 hr.

It appears that there were two distinct phases in the process of excretion in this insect, the first from 0 to 22 hr. and the second from 22 to 70 hr. The drops of urine collected during each phase were pooled and analysed.

0–22 hr., vol. 31 cu.mm.	Na 97 m.equiv.	K 79 m.equiv.
22–70 hr., vol. 16 cu.mm.	54 m.equiv.	144 m.equiv.

A sample of the blood mixture was laked and analysed and was found to contain Na 34 m.equiv., K 132 m.equiv. This case is further discussed in the next section.

(*d*) *Collection from the Malpighian tubules*

What is usually called 'urine' in *Rhodnius* would be more correctly called 'rectal fluid'. The fact that the concentration of potassium in the rectal fluid is greater than in the haemolymph does not automatically mean that the difference in concentration is established by the activity of the Malpighian tubules. Nevertheless, the relatively rapid rate of excretion and the relatively small surface exposed by the ampullae and rectal glands make it probable *a priori* that the Malpighian tubules play an important part.

In order to investigate the role of the Malpighian tubules it is natural that one should choose to work under conditions in which the composition of the urine differs substantially from that of the haemolymph, that is to say, at some time towards the end of the diuresis period or later. This was the first line of approach made to the problem. The insect was fixed to the operating table and an attempt was made (not always successful) to obtain a sample of urine by cannulation of the rectum. The abdominal tergites were then removed, a sample of haemolymph was taken and then collections were made first from the lower portion and then from the upper portion of the right-hand ventral tubule as already described. The results of five more or less successful experiments are given in Table 4.

The first point which clearly emerges is that in all cases except that of serial 12 the fluid in the upper portion of the tubule contains less sodium and much more potassium than the haemolymph. The differences are so great that the selective activity of the upper portion can hardly be in doubt. The samples taken from the lower portion all show a greater concentration of potassium and most show a smaller concentration of sodium than the haemolymph, but the differences are much less than in the case of the upper portion. This suggests that the differences in concentration established by the activity of the upper portion are degraded as the fluid passes through the lower portion, a somewhat surprising result if true.

These experiments were not altogether satisfactory for a variety of reasons. The operations were on the whole carried out too late in the course of excretion. Ideally one should choose a moment near the end of the diuresis period for the operation, but owing to the great variation in the duration of this period from one insect to another, the correct timing is a little difficult to achieve. In serial 12, for example, no urine had been passed at 7 hr. after feeding when the operation was begun; the

Table 4. *Collections from Malpighian tubules of naturally fed* Rhodnius

Serial	Temp. °C.	Time after feeding	Haemolymph			Urine			Upper tubule			Lower tubule		
			Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.
5	18–20	24·00	177	5	218	146	210	379	137	72	222	168	53	207*
6	18–20	24·00	188	7	221	220	163	392	86	122	227	140	67	218
8	18–20	26·00	164	11	182	—	—	—	98	133	212*	182	17	189 *
9	18–20	29·00	167	4	202	116	200	302*	134	90	251	144	63	182 *
12	18–20	7·00	149	6	179	—	—	—	188	12	251	202	13	262

Table 5. *Collections from Malpighian tubules of artificially fed* Rhodnius

Serial	Temp. °C.	Time after feeding	Haemolymph 1			Haemolymph 2			Upper tubule			Lower tubule		
			Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.
13	18–20	1·00	158	22	178 *	—	—	—	106	124	235	95	95	178*
14	18–20	0·30	153	12	184	156	11	185	57	147	204	82	109	168*
15	18–20	0·30	147	14	179	149	23	182	97	145	261	98	105	196*
16	18–20	1·15	140	23	182	140	33	184	55	162	198*	93	113	188*

The second sample of haemolymph belonging to serial 13 was lost through an accident.

* See text, p. 125, l. 26.

rectum was found to be highly distended with colourless urine in which there was hardly any sediment at all. When diuresis slackens and the tubules are no longer distended the difficulties of successful penetration are increased and the amounts of fluid obtained are less. There is also the possibility, envisaged by Wigglesworth, that fluid from the rectum may be drawn back into the lower portion of the tubule. In view of these disadvantages a second approach was made, taking advantage of the method of artificial feeding on ox blood with potassium chloride added. Under these conditions, even during the early part of the diuresis period, the urine had been found to contain substantially more potassium than the haemolymph. In this second series of experiments the insects were allowed to feed on a mixture of approximately 3 parts ox blood to 1 part of 171 mM. KCl, and two samples of haemolymph were taken, the first immediately after the insect had been opened up and the second at the end of the operation; this provided a check upon the possibility of changes taking place in the haemolymph during the operation. The results are presented in Table 5.

From the figures in Table 5 the following points may be noted:

(i) The changes in the haemolymph during the operation are negligible.

(ii) The fluid in the upper portion of the tubule contains less sodium (av. − 69 m.equiv.) and more potassium (av. + 127 m.equiv.) than the haemolymph; its osmotic pressure is greater (av. + 44 mM).

(iii) The fluid in the lower portion of the tubule contains less sodium (av. − 56 m.equiv.) and more potassium (av. + 88 m.equiv.) than the haemolymph; its osmotic pressure is practically identical with that of the haemolymph (av. + 2mM.).

(iv) The fluid in the lower portion of the tubule contains more sodium (av. + 13 m.equiv.) and less potassium (av. − 39 m.equiv.) than the fluid in the upper portion; its osmotic pressure is lower (av. − 42 mM.).

With the exception of the difference in concentrations of sodium between the upper and lower portions of the tubule these differences may be judged significant in relation to the errors of analysis. The difference in osmotic pressure between the fluid in the lower portion and the haemolymph is not statistically significant in relation to variations between individual insects.

In answer to the second question posed in the introduction it is therefore concluded that the upper portion of the tubule is able to elaborate a fluid containing less sodium and more potassium than the haemolymph, and that these differences in concentration become degraded during the passage of the fluid through the lower portion. These conclusions and other points arising from the experimental results are further discussed in the next section.

IV. DISCUSSION

The results presented in this paper are substantially in agreement with the work of Wigglesworth, but certain differences are to be noted.

First, the duration of the diuresis period in the present experiments was in general much longer than in Wigglesworth's experiments. The insects showed a tendency to retain the urine in the rectum, which was often considerably distended.

In many cases, both for naturally fed and artificially fed insects, a considerable amount of air was observed to be present in the stomach after feeding, and it seemed that the elimination of urine was more rapid when the amount of air was less. The arrangements for allowing the experimental insects to feed from a rabbit were the same as those used in rearing the whole culture, which was thoroughly healthy. There is no reason to believe that the slower rate of elimination was due to impaired physiological condition of the insect.

Secondly, Wigglesworth found that at the end of the diuresis period the urine contained only traces of sodium. In the present work the concentration of sodium in the urine of naturally fed insects was never less than half its concentration in the haemolymph. It has already been pointed out that the fluid in the lower portion of the tubule has generally the same osmotic pressure as the blood. This implies that the very much greater osmotic pressure of the urine in the later stages of excretion (when the yellow colour appears) is due to the activity of the ampullae and/or the rectal glands, and is in keeping with the observation that the urine must be retained in the rectum for several hours before these changes are manifested. In the three cases in which direct comparison is possible, serials 5, 6 and 9, Table 4, the potassium concentration in the rectal fluid was always very much higher than in the fluid taken from the lower portion of the tubule, whereas the sodium concentration was higher in one case and lower in the other two. The high osmotic pressure and high concentration of potassium in the yellow urine might be due to the secretion of potassium into it, but in view of the ability of the rectal glands of *Rhodnius*, and of insects in general (Wigglesworth, 1932), to remove water from the faeces and urine, it is probable that there is reabsorption of water from the rectum during the period in which the yellow colour develops. If this is granted then sodium must be reabsorbed at the same time, since the sodium concentration of the urine does not rise parallel with the osmotic pressure and potassium concentration; but there is no need to postulate the secretion of potassium into the rectum. On this view the reabsorption of water and sodium would in time produce a urine of higher osmotic pressure than the haemolymph and containing only traces of sodium. But there is no obvious explanation of why this seems to have happened in Wigglesworth's experiments and not in the present experiments.

The variation of the sodium/potassium ratio in the haemolymph of insects is very much greater than in other classes of animals as yet studied. Boné (1944) has shown that a high sodium/potassium ratio is characteristic of carnivorous and omnivorous insects, and a low ratio of herbivorous insects. Insects also seem able to tolerate very considerable alteration of their normal ratio. Tobias (1948), working on the cockroach, caused the ratio to fall from 6·2 to 2·7 by administration of potassium chloride through the mouth without apparent harm to the insect, and Roeder (1948) likewise found that the nervous system of the cockroach, bathed in simple salt solutions, could tolerate variations of the same order without impairment of function. In *Rhodnius* the value of the ratio found for fasting insects is about 16. After a normal meal it may rise to 50 or over. After a meal of blood with potassium chloride added it may fall to 1 or 2, and yet for several hours the insect continues to make

co-ordinated movements. The heart can continue to beat for 48 hr. with the ratio at 3.

In considering the ability of the Malpighian tubules and rectum to keep down the potassium concentration of the haemolymph after a normal meal, one must bear in mind that at the time when high concentrations of potassium appears in the urine the rate of urine flow is slackening and the rate of doing secretory work is not perhaps so great as appears at first sight. It is otherwise when the insect has fed on blood with artificially increased potassium content, and under these conditions the potassium concentration in the haemolymph is seen to rise. Further examination of the case of serial 19 (Fig. 4) brings to light some interesting points. In the laked blood upon which the insect had fed the sodium/potassium ratio was 0·26. During the first phase of excretion the sodium/potassium ratio of the urine was 1·23. Even if one assumes that all the potassium in the ox blood was contained in the corpuscles and that these remained intact, it is quite clear that during the first phase the excretion of potassium relative to sodium was not adequate to maintain the normal proportion of these elements in the haemolymph and tissues. Yet during this phase the ratio in the haemolymph was maintained at about the relatively high level of 10. Conceivably the tissues might regulate the haemolymph by taking up potassium as they were shown to do by Tobias, but in view of the relatively large amount of fluid ingested and the relatively small bulk of the tissues this is improbable. It is more likely that the potassium is retained in the stomach. Wigglesworth suggested that the increased excretion of potassium towards the end of the diuresis period was due to the fact that it was present mainly in the corpuscles and was only liberated as digestion proceeded. This may indeed be true, but one should bear in mind the possibility that the gut epithelium may exert a selective action upon the passage of ions through it. Certainly, the flooding of the haemolymph with potassium at the beginning of the second phase strongly suggests the breakdown of some form of restraint.

In the upper portion of the tubule the fluid contains less sodium and more potassium and has an higher osmotic pressure than the haemolymph. Since there is a net movement of water and solutes from the haemolymph into the lumen of the tubule we are bound to admit that the movement of potassium against the concentration gradient must be brought about by some active process involving the expenditure of energy. Sodium and water, on the other hand, move with their respective concentration gradients and these movements may be due to passive diffusion. In order to account for the secretion of urine by the upper portion of the tubule all we have to postulate is that potassium (together with some anion) is actively secreted into the lumen; this will raise the osmotic pressure of the tubule fluid, causing water to diffuse in, and the inward diffusion of water will set up a concentration gradient of sodium which will result in the passive inward diffusion of this ion.

In the lower portion of the tubule the composition of the fluid is more or less intermediate between that of the upper portion and that of the haemolymph. If we regard the cells of the lower portion as freely permeable to all the substances in question than all the changes can be accounted for on the basis of passive diffusion alone. But there is one very good reason why this simple explanation cannot be

accepted. Since the fluid coming from the upper portion of the tubule has an higher osmotic pressure than the haemolymph, passive diffusion of water would result in an increase in volume of the urine as it passed through the lower portion, but Wigglesworth's work suggests strongly that the reverse is true, that there is reabsorption of fluid into the haemolymph from the lower portion of the tubule. It is more likely that the cells of the lower portion are actively concerned in the reabsorption of water from the urine into the haemolymph; the osmotic forces involved, however, appear to be small. Whether or not there is reabsorption of water, it is at least certain that water does not pass from the haemolymph into the urine in the lower portion; and therefore if the potassium concentration of the urine falls in the lower portion, it follows that potassium must move from the urine into the haemolymph. Whether sodium moves inward or outwards cannot be decided until something is known about the changes in volume of the urine as it passes through the lower portion. One would also like to know more about the formation of hypotonic urine during the early stages of diuresis after a normal meal; unfortunately no collections were made from the Malpighian tubules of naturally fed insects during the early stages of diuresis.

The foregoing suggestions as to the mechanism of urine formation are regarded as no more than an attempt to apply the principle of economy of hypothesis to the available data.

The bearing of the present experiments upon Wigglesworth's theory of uric acid excretion is quickly dealt with. Wigglesworth's theory demands that sodium and/or potassium be secreted into the upper portion of the tubule and reabsorbed from the lower portion. This is precisely what has been found in the case of potassium; the question of reabsorption of sodium is still undecided.

There is just one other small matter which may be relevant. If a fluid contains only sodium and potassium ions with the equivalent number of monovalent anions, fully dissociated, then according to the conventions used in this paper $Na+K=O.P.$ If $Na+K<O.P.$, other osmotically active substances must be present, if $Na+K>O.P.$ then the cations must be in balance with polyvalent anions or dissociation must be incomplete. In Tables 2, 4 and 5 certain figures for O.P. are marked with an asterisk; these are cases in which $Na+K>O.P.$ There are 13 such cases altogether, one under haemolymph, three under urine, two under upper tubule and under lower tubule there are seven cases out of the total of nine samples of fluid taken from the lower tubule. Some of the discrepancies are well within the limits of experimental error. But taking the four cases in Table 5 the average value of $Na+K-O.P.$ for the lower tubule is 15 which is statistically significant ($P=0{\cdot}05-0{\cdot}01$) in relation to variation between individuals and which, being based upon at least eight observations for each element, is probably just significant in relation to the errors of analysis. What this means is hardly worth speculating upon at present; but it is an observation which may one day fit into place in a more detailed account of the mechanism of excretion.

Other work relating to the excretion of salts and water by Malpighian tubules was discussed in a recent paper (Ramsay, 1951).

SUMMARY

1. The very considerable changes in the concentrations of sodium and potassium in the urine of *Rhodnius* during the normal course of excretion are not reflected in the haemolymph which remains relatively constant in composition.

2. The concentration of potassium in the haemolymph can be increased some tenfold by adding potassium chloride to the blood on which the insect feeds. Such increase in the concentration of potassium can be tolerated for several hours without loss of nervous co-ordination, but it is ultimately fatal.

3. The upper portion of the Malpighian tubule can secrete a urine which contains more potassium and less sodium than the haemolymph.

4. During the passage of this urine through the lower portion of the tubule the differences in concentration between urine and haemolymph became reduced.

5. It is probable that water and sodium, but not potassium, are reabsorbed from the urine in the rectum during the later stages of excretion.

I wish to thank Dr Wigglesworth for reading the manuscript of this paper and for most valuable discussion.

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THE INFLUENCE OF WATER AND LIGHT UPON THE COLOUR CHANGE OF SIGHTLESS FROGS (*RANA TEMPORARIA*)

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INTRODUCTION

Chromatic behaviour in the frog (*Rana temporaria*) is influenced by a number of physical factors including light and moisture. The changes observed are especially related to the activity of the dermal melanophores. Thus a white illuminated background (light-scattering) or dryness induces the contraction of the melanophores giving pallor, whereas a black illuminated background (light-absorbing) or moisture induces the expansion associated with darkening.

As regards moisture, Hogben (1942), summarizing the evidence then available, concludes (*a*) low humidity beyond a certain limit either abolishes the primary response to a light or inhibits the stimulation of the B-area of the eyes, (*b*) the action of humidity almost certainly involves the skin receptors.

Since the primary response is presumed to reappear in the adult with the elimination of vision, an investigation of the effect of moisture upon melanophore activity in sightless frogs was needed.

MATERIAL AND METHODS

Medium to large specimens (15–35 g.) of *R. temporaria* were used throughout the experiments. They were fed weekly with liver or lean horse-meat.

One of the three following techniques was used to blind the frogs after anaesthetization by injections of 0·5 c.c. of 15% urethane into the dorsal lymph space.

A. *Cauterization of the optic chiasma*

The frog was placed with its ventral side uppermost on a dissecting board and held in position with tapes across the fore- and hind-limbs. The mouth was opened as wide as possible and fixed by a clip. A median longitudinal incision was made in the mucous membrane of the roof of the mouth, which was then drawn apart and held by a second clip so as to expose the anterior half of the floor of the skull. A small hole was bored with a dental drill in the parasphenoid at the level of the optic chiasma which is visible through the bone. An electric cautery needle was then applied to the central region of the optic chiasma so as to sever the connexions of the optic nerve to the brain. The mucous membrane was then replaced, the mouth closed and the frog allowed to recover in gently running water.

B. *Severing of the optic nerve*

The frog was attached to the dissecting board as in the previous operation, and the median longitudinal slit in the mucous membrane drawn aside to reveal the band of muscles, including the optic nerve, running from the side of the orbit to the eyeball. This band was severed close to the eyeball on each side.

C. *Elimination of the eye itself as an organ of vision*

The eye was put out of action by either of the two following methods: (1) cauterizing the eyeball internally to destroy the retina, after exposing it by drawing aside the mucous membrane of the roof of the mouth, the frog being placed on the dissecting board as before, or (2) the destruction of the lens and retina by the external application of the cautery needle through the pupil.

Except in the last group, the frogs were not fed for a fortnight following the operation so that healing of the mucous membrane could proceed far enough to allow of forced feeding without further injury.

The recovery in all was complete, and as a result of the operation there was no appreciable increase in the mortality rate compared with normal intact animals.

A bench was illuminated by four 60 W. pearl bulbs fitted with reflectors 27 cm. in diameter hanging 45 cm. from the bench, for studies of the reactions to illuminated backgrounds, and a dark chamber was used for reactions in the absence of light.

To provide the white or black backgrounds the whole inside of bins or jars used as containers was painted with white enamel (light-scattering) or black paint (matt finish, light-absorbing).

In between experiments, operated frogs were kept in small groups so that individual recognition by their pattern was possible throughout the investigation. They were retained in green enamel bins fitted with a lid and tilted so that half the floor was covered with water—the 'dark moist' condition.

In the experiments three conditions were used with respect to moisture—'dry' 'half-immersed', and 'immersed'. The frogs were placed singly in either black- or white-walled jars (7 cm. in diameter and 12 cm. in depth) with gauze tops secured by an elastic band, for the first two of these conditions.

(*a*) 'Dry' conditions—surface moisture was removed from the frogs which after evacuation of the bladder were placed in dry jars.

(*b*) 'Half-immersed' conditions—the frogs were placed in jars with sufficient water added to cover the hind-limbs and posterior half of the dorsal surface.

(*c*) 'Immersed' conditions—frogs were placed in groups of up to ten in a large white enamelled bin ($28 \times 41 \times 33$ cm.), filled with water to a depth of 18–20 cm.

The temperature of the water was maintained in all cases the same as that of the dry jars.

The condition of the dermal melanophores in the web of the hind-limb was observed under the microscope and the degree of expansion assessed numerically using the melanophore index scale of Hogben & Gordon (1930), 1·0 indicating the contracted condition and 5 the reticulate expanded condition.

OBSERVATIONS

All specimens prior to operation showed the appropriate responses to the various combinations of experimental conditions as follows:

White illuminated dry (WID): $\mu = 1–2$.
White illuminated immersed (WII): $\mu = 2{\cdot}5–5$.
Black illuminated dry (BID): $\mu = 5$ (tendency for some contraction with time).
Black illuminated half-immersed (BI½I): $\mu = 5$.
Dark dry (DD): $\mu < 5$ (variable; tendency to paleness with time).
Dark half-immersed (D½I): $\mu = 5$ (some variability).

After complete recovery from the operations the animals were subjected again to the same combinations of experimental conditions and the melanophore responses of each individual noted.

A. *Cauterization of the optic chiasma* (*October–December*, 11 *males*, 13 *females*)

This operation caused permanent darkening ($\mu = 5$) whatever the environmental conditions. The melanophore reactions were tested repeatedly for 5 weeks in white illuminated dry and half-immersed, black illuminated dry and half-immersed, and dark dry and half-immersed environments for periods of 6–8 hr. Occasionally after 6 hr. in dark dry conditions a few specimens showed slight paling more marked macroscopically than microscopically, the melanophores never showing more than a slight contraction from the reticulate condition. Under all other conditions the melanophores remained fully expanded.

After 5 weeks the eye was destroyed in half the operated animals by the method of external cauterization.

This operation resulted in no change in the condition of the dermal melanophores which remained fully expanded in all environments.

B. *Severance of the optic nerve*

Operations were performed in November, January and May (Tables 1–3). In general there was a loss of black-background response. In a dry environment, irrespective of background or illumination, the melanophores contracted. The addition of moisture caused some expansion of the melanophores with resultant darkening.

In the November and January series (Table 1 and 2) the reactions of the melanophores to the various environmental conditions were tested repeatedly over a period of 2 months, during which time some variation in reaction was noted. The maximal and minimal values of the melanophore index are therefore given in Tables 1 and 2 for each combination of conditions used.

Nos. 7 and 8 (Table 1) died after 5 weeks, and in both cases several days before death, while still apparently healthy, there were no chromatic responses, and the melanophores remained permanently expanded under all conditions.

No. 2 (Table 2) is peculiar in showing very little chromatophore activity, particularly in the last month. Contraction of the melanophores was observed only in dark

Table 1. *First series (November–January): optic nerve severed*

Males	DM (18 hr.)	WID (2–6 hr.)	WI½I (4–6 hr.)	BID (2–6 hr.)	BI½I (4–6 hr.)	DD (2–6 hr.)	D½I (4–6 hr.)
1	2 to 2·5	1·5 to 2·5	2·5 to 3·5	1·5 to 2	2·5 to 3·5	1 to 1·5	3−
2	1·5 to 2+	1+ to 3·5	3 to 4+	1+ to 3·5	3+ to 3·5	1+ to 3	2 to 3+
3	1·5 to 3	1·5 to 2	2+ to 3·5	1·5 to 2·5	2 to 3+	1·5 to 2·5	3 to 3+
4	2− to 2+	1 to 1+	2	1 to 1+	1+ to 2·5	1 to 1+	2 to 2+
5	2 to 3	1·5 to 2	2 to 2·5	1 to 2·5	2+ to 3+	1 to 2+	2+ to 5−
6	2 to 3	1 to 2−	2+ to 4	1 to 1·5	2+ to 3·5	1 to 2	2 to 3
7	3 to 5	1·5 to 5−	3 to 5	2− to 2·5	2+ to 4·5	1+ to 2−	2·5 to 4
8	3 to 5	1+ to 1·5	3	1+	3 to 4+	1+	3 to 5
9	3 to 4·5	1+ to 2	3 to 4	1+ to 2	3·5 to 4·5	1 to 2	3 to 4·5

Table 2. *Second series (January–April): optic nerve severed*

Males	DM (18 hr.)	WID (6 hr.)	WI½I (6 hr.)	BID (6 hr.)	BI½I (6 hr.)	DD (6 hr.)	D½I (6 hr.)
1	1 to 4	1	2+ to 3	1 to 1+	2 to 3	1	1+ to 3
2	2·5 to 5	4·5 to 5	5	4 to 5	5	2 to 5−	2 to 5
3	3 to 5	1 to 1+	2·5 to 3	1	1·5 to 5−	1	1+ to 4·5
4	1·5 to 5	1	2 to 5−	1	3− to 5	1	1 to 2·5

conditions although under dry conditions, irrespective of the background, there was a very marked macroscopic paling.

The remaining examples (Table 2) show even more clearly than the November series the very marked and rapid contraction in dry conditions (maximum paling being achieved in the first hour), irrespective of background or illumination.

The observations in Table 3 are a sample of the group operated upon showing the range of variation between individuals. They were recorded a week after recovery and occupied six consecutive days. Between daily experiments the frogs were stored in the usual tilted bins, but the lid was left ajar to keep the specimens cool.

Here again in this series, the rapid contraction under dry conditions is evident, whilst the addition of moisture shows that some expansion occurs.

The two groups were separated on the basis of response to white illuminated immersed conditions before the operation. In the first, expansion was maxima within 2 hr., whereas in the second it was slight to intermediate. After severance o the optic nerve however, the two groups showed the same range of variation ir response to moist conditions, some specimens having a much smaller degree o expansion than others.

C. *Elimination of the eye as an organ of vision*

(1) *Destruction of the retina by internal cauterization*

The results are tabulated in Table 4 and again show the range of index variatio in the individuals noted during the two months of repeated observation.

No. 8, which survived the operation for 5 weeks, had chromatic responses com parable to the remainder for the first 4 weeks, but in the last week no chromati response was observed, the melanophores being permanently expanded.

Table 3. *Third series (April–May)*: *Temp.* 13·5–15° C. *Optic nerve severed*

(*a*) Males and spawned females which prior to operation had the melanophores expanded completely within 2 hr. of being immersed on a white illuminated background, i.e. WID→WII (2 hr.): *μ*, 1–2→5.

Males

	0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.	
Shady moist→BID	2·5	1	1	1													
Shady moist→DD	3·5	1	1	1			5	3	3	2+			5	1	1	1	
Shady moist→WID	3	1	1	1			5	2	1	1+			4·5	1	1	1	
							5	4	3−	2+			5	1·5	1+	1	
	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.
SM→BID (1 hr.)→BI½I	4−	1	1+	1+	1	1+	5	2+	4	5−	4·5	3·5	4−	2	5	2+	5−	4·5
SM→DD (1 hr.)→D½I	3−	1+	1	1	1	1	5	2·5	5	5	5	5	5−	2−	3+	3+	4−	3·5
SM→WID (1 hr.)→WI½I	3	1	1	1	1	1	5	2+	4+	4	4+		5	1·5	2·5	4	3−	4

Females

	0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.		
Shady moist→BID	3+	1+	1+	1			4·5	1	1	2								
Shady moist→DD	3+	1+	1+	1+			3·5	1	1	2−			5	1·5	1·5	1+		
Shady moist→WID	3+	1·5	1+	1			4·5	1·5	1+	1			3	1·5	1·5	1+		
							4·5	1·5	1+	1			5	2·5	1·5	1+		
	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.
SM→BID (1 hr.)→BI½I	4	1+	2−	2	2		5	2	5−	5	5		5		4+	3·5		
SM→DD (1 hr.)→D½I	4+	2−	2−	3+	2	2	5	2−	3	5−	4·5	5	5	3	4+	4+	3−	3−
SM→WID (1 hr.)→WI½I	3+	1·5	1	1+	1·5	2+	4·5	1·5	4+	4+	5	5	5	2·5	2+	2·5	3·5	4+
														2·5		2·5	2+	2·5

Table 3 (*cont.*).

(*b*) Males and spawned females which prior to operation had incomplete expansion of the melanophores on being immersed on a white illuminated background, i.e. WID→WII (2 hr.): μ, 1−2→2−3·5.

Males

	0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.		
Shady moist→BID	2	1	1	1			5	1·5	1·5	1·5			4−	2+	2·5	2·5		
Shady moist→DD	2−	1	1	1			2	1·5	1·5	1·5			2+	2·5	2·5	2·5		
Shady moist→WID	2	1	1	1			5−	2+	1+	1+			2	3+	2+	2+		
	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.
SM→BID (1 hr.)→BI½I	2	1	1	1	1	1	4·5	1·5	3−	4−	4·5	3+	3	3	3−	3·5	4+	3·5
SM→DD (1 hr.)→D½I	2·5	1	1	1·5	2−	2+	5−	2−	3	2·5	4+	4·5	4	2+	4	3+	2·5	3
SM→WID (1 hr.)→WI½I	2	1	1	1	1+		5	2−	3·5	3·5	3−		2	2+	3	3	4	

Females

	0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.			0 hr.	1 hr.	2 hr.	4 hr.		
Shady moist→BID	2	1	1	2−			5	5	5	2·5			4	2−	2·5	2		
Shady moist→DD	3−	1·5	1·5	1·5			3	1	1	1			2·5	2−	2−	2−		
Shady moist→WID	2+	1·5	1+	1+			5	5−	2+	2+			3	2	2−	2−		
	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1 hr.	1 hr.	3 hr.	5 hr.	7 hr.
SM→BID (1 hr.)→BI½I	3	2−	2·5	3·5	4−	3	4+	2	1+	1	3·5	3	3	2+	3	2	2·5	2
SM→DD (1 hr.)→D½I	2·5	1+	2·5	2·5	2+	2+	5−	2+	4	3	4·5	5−	5−	2·5	3+	3+	3	3
SM→WID (1 hr.)→WI½I	2+	1·5	3−	2·5	3		5	2+	2+	3·5	4·5		3	2	2·5	2·5	2+	

Table 4. (*October–December*)

Males	WID (6 hr.)	WI½I (6 hr.)	BID (6 hr.)	BI½I (6 hr.)	DD (6 hr.)	D½I (6 hr.)
1	2 to 4−	3·5 to 5	2−	4·5 to 5	2+ to 3+	3·5 to 5
2	2·5	3·5 to 5	2−	5	2	5
3	2− to 2+	2 to 3	2·5	5	2+	2·5 to 5
4	1+ to 4	3·5 to 4+	2+ to 3	5	2 to 2+	2·5 to 5
5	1+ to 2+	3·5 to 5	2− to 2·5	4 to 5	1+ to 2−	1+ to 2·5
6	3−	5	2·5	5	1·5 to 3	4
7	2+ to 3+	3− to 4	2− to 3+	3+ to 4·5	2 to 2·5	2·5 to 4
8	2·5	4+	4	4	2·5 to 3	3 to 4·5
9	2+ to 4−	4·5	2·5 to 4	5	1·5 to 2·5	2·5 to 4+

Thus, in general, the melanophores of this group were more expanded than in those specimens with the optic nerve severed. There was a lack of the extreme contraction shown in the previous groups, but dry conditions did induce the contraction of the melanophores, whilst the addition of moisture gave fairly consistent expansion, background and illumination having no obvious effect.

(2) *Destruction of the lens and retina by external cauterization*

The results are shown in Table 5.

Table 5. (*January–April*)

Males	DM (18 hr.)	WID (2–6 hr.)	WI½I (4–6 hr.)	BID (2–6 hr.)	BI½I (4–6 hr.)	DD (2–6 hr.)	D½I (4–6 hr.)
1	1 to 5	1	1 to 5−	1	1+ to 4·5	1	1 to 3·5
2	1 to 3·5	1	4−	1	2·5	1	5
3	2− to 5	1 to 2·5	3 to 5	1+ to 2·5	3+ to 5−	1 to 2−	3·5 to 5
4	2+ to 5	1	5	1	2·5	1 to 2−	5
5	3·5 to 5	2 to 3+	4·5	2+ to 4	5− to 5	3+ to 4	5
6	1 to 2·5	1+ to 2+	2 to 2·5	1+ to 2+	1+ to 2·5	1 to 2−	1 to 2·5
7	4 to 5	4·5 to 5	5	5	5	3+ to 5	3·5 to 5

Nos. 2, 4 and 5 died before the end of February, and again all failed to show any chromatic response (the melanophores being fully expanded) several days before death.

No. 7 is unusual in showing throughout little change in the melanophores; only in darkness did any appreciable contraction occur.

But on the whole, irrespective of background and illumination the dermal melanophores contract in dry conditions—the contraction being achieved in 1–2 hr. The addition of moisture causes an expansion of the melanophores.

A second group (ten males, December-February) in which the eye was cauterized externally gave extremely variable results to moisture and illumination. Not only did the responses of different individuals show great divergences, but the same individual showed widely different responses in successive experiments. In dark moist storage conditions there was a marked tendency for expansion, more particularly complete in the latter part of the period. However, again there were definite indications that irrespective of background or illumination, in dry conditions the melanophores were more contracted than in moist conditions.

The observations can be summarized as follows:

(1) Cauterization of the optic chiasma eliminates all chromatic response to both light, background and moisture, the melanophores being permanently expanded.

(2) The prevention of the eye functioning as an organ of vision by damage or by severance of the optic nerve results in an elimination of the response to light and background alone. The response to the presence (expansion) or the absence (contraction) of moisture remains.

DISCUSSION

The permanent expansion of the melanophores resulting from the cauterization of the optic chiasma indicates that the damage produced had disturbed the connexions in the mid-brain or infundibular stalk. Thus Geiringer (1937) found that in *Hyla arborea* injury to the mid-brain brought about the permanent expansion of the melanophores. Also, Etkin (1941) observed that lesions of the infundibulum induced permanent darkening in tadpoles. It is generally agreed that under appropriate conditions (white illuminated background), the eye produces inhibitory impulses which, passing through the supra-optico-hypophyseal tract of the infundibular stalk, prevent the secretion of the pars intermedia, and hence there is a contraction of the melanophores. Further, it is usually accepted that when the nervous connexion between the eye and the pars intermedia is severed, the pars intermedia, freed from restraint, secretes the melanophore factor continuously with resultant permanent expansion.

Parker (1948), reviewing the results obtained from blinded amphibians, says: 'Thus a general rule appears to hold that amphibians when blinded become somewhat dark in tint, lose most of their former capacity to respond to the illumination of their surroundings but still respond to it by being a little darker in bright light than in darkness.' This residual response is believed to be the primary response which in normal adults is overcome by the opposing secondary ocular response.

However, from the observations recorded here, it is apparent that this statement needs at least some qualification in the species in which moisture is a significant variable in relation to the colour changes of the intact individual. It has been shown (Rowlands, 1950) that moisture is a significant factor in the pigmentary changes of *Rana temporaria*, the presence of water causing the expansion of the dermal melanophores irrespective of light and background, whereas the absence of water induces contraction.

These responses are seen to remain when the ocular responses to light and background are eliminated. Whereas in the normal frog a protracted length of time is needed for absence of moisture to overcome the ocular darkening response to a black illuminated background, in the blinded individual, a rapid contraction occurs when water is removed.

Hogben's second conclusion that the action of humidity almost certainly involves the skin receptors would seem to be justified, since blinded frogs, like normal ones, respond dermally quite independently of light and darkness in situations which are dry or wet.

In a previous discussion (Rowlands, 1950) of Hogben's statement that low humidity beyond a certain limit either abolishes the primary response to light or inhibits the stimulation of the B-area of the eye, it was shown that the response due to moisture must be considered as distinct from the primary response, since the two responses could be made to antagonize or reinforce each other. If the primary response is extant in adult frogs it does not become significant even when the secondary ocular response is removed (i.e. blinded), as it is overridden by the effect of moisture. In general, there were occasional indications that in darkness the melanophores were more contracted than when illuminated (primary response), but in the majority of cases illumination had no significant darkening effect.

If the permanent expansion obtained when the infundibular stalk was damaged in cauterizing the optic chiasma was due solely to a break in the nerve tracts from the eye to the pars intermedia, the results from individuals in which the optic nerve had been severed should have been identical. This was not so, however, for in the first case all chromatic response, both dermal and ocular (with the exception of a very slight indication of some paling in darkness—primary response) is eliminated, whereas in the second, ocular responses alone are eliminated.

Operated specimens of the second group dying before the end of the experiments (Table 1, nos. 7 and 8; Table 4, no. 8; Table 5, nos. 2, 4 and 5) lost their capacity of reacting to moisture and became, as in the first group, permanently expanded. Possibly degeneration of the optic nerves reaches the chiasma and affects the tracts of the infundibular stalk.

The colour response to moisture is effective over the whole skin and therefore would imply the control by some regulatory mechanism which could be nervous or hormonal, rather than a direct effect of moisture upon the melanophores or a simple reflex action. The regulatory mechanism may operate through the infundibular stalk; in fact, any nerve impulses entering the pars intermedia pass through the infundibular stalk. These impulses from the skin to the posterior lobe are apparently checked together with the impulses from the eye when the infundibular stalk is damaged, resulting in the permanent expansion of the melanophores.

On the other hand, the pars intermedia may not be directly concerned with effects relating to moisture. It has been suggested that the colour change is associated with the conservation of moisture and temperature control. A pale skin will reflect light, hence heat, and so conserve moisture. If so, then it would be reasonable to suppose that it is the contraction of the melanophores which is the active adaptive feature, and in the presence of water, this adaptation being unnecessary, the melanophores expand. It may be that the response to presence or absence of moisture is associated with the secretion of adrenaline, which is known to cause the contraction of the melanophores. Since damage to the infundibular stalk eliminated dermal responses, if we have only a single hormone system in colour change as advocated by Parker & Scatterty (1937) and Etkin (1941), then in dry conditions inhibition impulses must be transmitted from the skin to the pars intermedia via the infundibular stalk. If, however, we have in the frog a two-hormone system as deduced by Hogben & Slome (1931), then the infundibular stalk must be associated with the paling response

directly or indirectly through the adrenals, in which case it may well be that any dermal responses to moisture would be overcome by the excessive secretion of the pars intermedia when the infundibular stalk is disturbed, rather than the disturbance of any tracts from the skin to the pars intermedia.

SUMMARY

1. Cauterization of the region of the optic chiasma causes the permanent expansion of the melanophores. Subsequent cauterization of the eye has no further effect.

2. Severance of the optic nerve or damage to the eye itself results in the cessation of the chromatic response to illumination and background.

3. Specimens operated upon in this manner do not lose the melanophore response to moisture, contracting in dry and expanding in moist conditions.

4. Only a slight residual primary response can be detected.

5. The implications of these results in relation to chromatic control are discussed.

I should like to thank Prof. E. A. Spaul for his constructive criticism of the paper.

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FUNCTIONAL SIGNIFICANCE OF THE NEUROSECRETORY BRAIN CELLS AND THE CORPUS CARDIACUM IN THE FEMALE BLOW-FLY, *CALLIPHORA ERYTHROCEPHALA* MEIG.

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(With Ten Text-figures)

CONTENTS

I. THE PROBLEM

Neurosecretory cells, i.e. nerve cells which cytologically show signs of a secretory activity, are found in many animals. In vertebrates they were described originally by Dahlgren (1914) and Speidel (1919, 1922) in the hindmost part of the spinal cord of rays and teleosts. In 1928 E. Scharrer independently discovered neurosecretory cells in the nucleus pre-opticus of the hypothalamus of the teleost *Phoxinus laevis*. Since then neurosecretory cells have been recorded in a number of different animals. The investigation of neurosecretory cells has been mainly carried out by E. & B.

Scharrer and their collaborators, and by Hanström. Reviews of the literature on the occurrence of neurosecretory cells and on the problem of neurosecretion were given by E. & B. Scharrer (1937, 1944, 1945). The first description of neurosecretory cells in invertebrates is due to Hanström (1931, 1933, 1934*a*, *b*, *c*), who found secreting nerve cells, the so-called 'Organ X', in the central nervous systems of different Crustacea. The description of the neurosecretory cells in cephalopods by Young (1936) must also be mentioned.

In insects, neurosecretory cells were first described in the brain of the honey-bee by Weyer (1935). Later they have been found in several orders.

Neurosecretory cells in insects are found not only in the brain but also in the frontal ganglion, the sub-oesophageal ganglion and in some abdominal ganglia (Day, 1940*a*, *b*; B. Scharrer, 1941*a*).

As neurosecretory cells have been found in annelids, crustaceans, xiphosurans, insects, molluscs and, among the vertebrates, in fishes, amphibians, reptiles and mammals, including man, one must suspect that the function of these cells is probably concerned with some fundamental physiological process. However, very little is known of the functional significance of these cells.

The first evidence of one function of neurosecretory cells was provided by Wigglesworth (1939, 1940), who showed that in the insect *Rhodnius prolixus* a factor regulating moulting is produced by the pars intercerebralis of the protocerebrum, in which part of the brain Hanström (1938) had discovered neurosecretory cells.

Later, Williams (1946, 1947, 1948*a*, *b*), studying the diapause of the moth *Platysamia cecropia*, found that the neurosecretory cells of the brain produce an over-all controlling factor for the regulation of diapause.

The investigations of Wigglesworth and of Williams thus revealed the functional significance of the neurosecretory cells of the brain during the development of insects (see also the discussion, p. 164).

Further, Brown & Cunningham (1941) have demonstrated that in *Limulus* the chromatophorotropic principle of the nervous system shows a distribution, which can be correlated with the distribution of the neurosecretory cells described by B. Scharrer (1941*b*) within the central nervous system of the same species.

Quite recently Knowles (1951) reported the occurrence of fuchsinophile cells, probably neurosecretory, in the post-oesophageal commissure of *Penaeus braziliensis* and in the nerves from this commissure to the 'sinus-plate'. Extracts of the commissure and the nerves concentrate the red and white pigment as does extract of the sinus-plate.

An indication of the functional significance of the neurosecretory cells in the hypothalamus of mammals is given in a paper by Ranström (1947), who sets forth as a working hypothesis that the neurosecretory cells may be involved in the regulation of sleep.

The following investigation was started in order to elucidate the function of the neurosecretory cells of the brain of the adult female *Calliphora erythrocephala*.

As will be mentioned in detail in the next section, the corpus allatum is probably innervated from the neurosecretory cells of the brain, and so experiments were first

carried out to see if removal of the neurosecretory cells had an effect like that of allatectomy (cp. E. Thomsen, 1940, 1942). A preliminary paper was published by the author in 1948 (E. Thomsen, 1948*a*).

During further investigation it became clear that the function of the neurosecretory cells is not limited to the probable activation of the corpus allatum, but is in all probability concerned with other fundamental physiological processes. This will be shown in the present paper. The connexion between the neurosecretory cells of the brain and the corpus allatum is established via the corpus cardiacum, and consequently the function of the corpus cardiacum, which has previously been found to produce a hormone (Brown & Meglitsch, 1940; Hanström, 1940; M. Thomsen, 1943), was also studied.

All the flies used in the experiments have been kept at a constant temperature of 25° C.

II. NEUROSECRETORY CELLS IN THE BRAIN AND THEIR CONNEXION WITH THE CORPUS CARDIACUM AND THE CORPUS ALLATUM

In adult females of *Calliphora* four groups of neurosecretory cells are found in the brain (Fig. 1). Two of these groups, the *medial groups*, are situated near each other

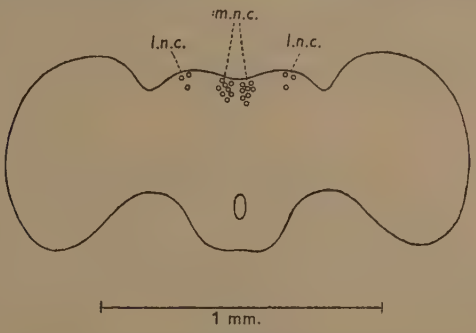


Fig. 1. Diagram of the brain of the adult female *Calliphora erythrocephala*, posterior view. *l.n.c.* lateral neurosecretory cells; *m.n.c.* medial neurosecretory cells.

in the pars intercerebralis of the protocerebrum, each group comprising about eight cells. The two other groups, the lateral groups, consisting each of three cells, are situated in the lateral parts of the protocerebrum. Lateral groups of neurosecretory cells in the insect brain were for the first time described by Cazal (1948) and Williams (1948*a*) simultaneously. (A description of the cytology of the neurosecretory cells of *Calliphora* will be given later in a special paper by M. Thomsen.)

The nerves which originate from the neurosecretory cells have not yet been traced within the brain in *Calliphora*. The course of the nerves outside the brain was described by Hanström (E. Thomsen, 1942, p. 332, Text-fig. 1). In accordance with investigations on other *Cyclorrhapha* made by Cazal (1948) it is assumed that each of the two nerves leaving the brain represents the fused nervus internus and externus

(also called the nervus corporis cardiaci I and II). The nervi interni originate from the two medial groups of neurosecretory cells, the nervi externi take their origin from the two lateral groups of neurosecretory cells. The two nerves (in the figure named *n.c.c.* I) soon unite and fuse with the nervus recurrens to form a single nerve. Just before its entrance into the fused corpus cardiacum and the ganglion hypocerebrale (=ganglion ventriculare of Cazal) this nerve divides into two branches. From the corpus cardiacum+ganglion hypocerebrale two nerves, the nervi allati, go to the unpaired corpus allatum (E. Thomsen, 1942, Pl. XXXVI, fig. 4). Presumably, as in most other insects, the nervi allati branch off from the nervi interni and externi.

This morphological connexion renders it highly probable that the normal function of the corpus allatum is to some degree controlled by the centres in the brain from which these nerves originate.

III. THE FUNCTION OF THE MEDIAL NEUROSECRETORY CELLS

A. *Allatectomy of females*

As mentioned above I originally started the experiments on the hypothesis that the function of the neurosecretory cells might be an activation of the corpus allatum. If this held true a removal of the neurosecretory cells should have an effect similar to that of allatectomy. (In the following the terms medial neurosecretory cells and lateral neurosecretory cells will be abbreviated to m.n.c. and l.n.c.)

Table 1

Age of flies at 25° C.	Length of egg-chambers in mm.									
	Series 1						Series 2			
1 day	—	—	—	—	—	—	0·08	0·06	0·06	0·05
2 days	0·09	0·10	0·08	0·08	0·10	0·08	0·08	0·10	0·15	0·13
3 days	0·16	0·16	0·12	0·13	0·16	0·16	0·18	0·20	0·13	0·14
4 days	0·38	0·40	0·41	0·11	0·30	0·15	0·30	0·40	0·43	0·18
5 days	0·31	0·23	0·56	0·75	0·25	0·30	0·13	*1·48*	0·75	0·30
6 days	*1·48*	0·36	0·55	1·23	0·56	0·43	1·25	1·13	0·68	1·13
7 days	1·18	0·30	0·55	0·85	*1·38*	*1·52*	*1·48*	*1·38*	*1·46**	*1·48**
8 days	*1·30*	R.E.*	R.E.*	R.E.	R.E.	R.E.	—	—	—	—

Ripe eggs are printed in italics.
* Eggs had been deposited on the same day.
R.E. = ripe eggs (not measured).

(1) *Effect on ovaries.* In former experiments (E. Thomsen, 1942) it was found that if young females less than 8 hr. old (25° C.) were deprived of their corpora allata, the ovaries in most cases only developed to the point at which the deposition of yolk begins. The effect on the size of the ovaries of the removal of the neurosecretory cells was therefore tested. In the previous experiments the allatectomized females were kept for 14 days (25° C.) after the operation; but since, it turned out that the mortality of flies deprived of their neurosecretory cells rose to nearly 80% after 14 days as compared with 60% after 7 days, a new series of experiments was performed, in which the allatectomized females were kept for only 7 days after the

operation. The normal development of the ovaries of *C. erythrocephala* is shown in Table 1 (which is taken from E. Thomsen (1942)). It is seen that at a temperature of 25° C. the flies generally possess ripe eggs on the 7th day. A normal ripe egg on an average measures 1·44 mm., and the deposition of yolk begins when the egg-chamber reaches a length of 0·25–0·30 mm. (For a detailed description of the morphology of the ovaries and their development see E. Thomsen, 1942.)

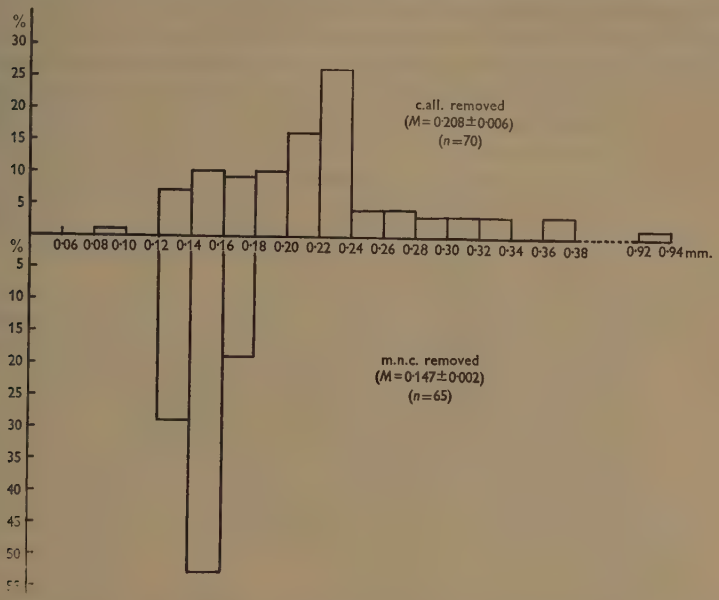


Fig. 2. Ordinate: percentage of flies. Abscissa: length of egg-chambers in millimetres of allatectomized females (c.all. removed) and of females deprived of their medial neurosecretory cells (m.n.c. removed).

Altogether eighty-four females less than 8 hr. old had their corpora allata removed. A very small piece of the side lobes of the ring-gland was excised together with the corpus allatum, the greater part of the side lobes being left intact.* The flies were kept in the usual way (E. Thomsen, 1942, p. 341) and fed on dog meat, sugar and water. Seventy of the flies survived, the mortality thus being 17%. The flies were dissected under Ringer's solution and the biggest egg-chambers were measured. The lengths in millimetres of the egg-chambers of these flies are given in Fig. 2 (upper curve).

* In my paper of 1942, I did not regard the corpus allatum of the adult fly as a part of the ring-gland. This was unfortunate. The ring-gland of a newly emerged fly comprises the corpus allatum, the side lobes and the corpus cardiacum fused with the ganglion hypocerebrale. The side lobes, homologous with the 'pericardial glands' of lower insects (E. Thomsen, 1941), degenerate during the first three days of adult life, but the corpus allatum and the corpus cardiacum persist. So it is only in the newly hatched fly that one can actually speak of the ring-gland.

In accordance with the previous experiments it was found that the ovaries of allatectomized females generally develop only to the stage at which the deposition of yolk begins. *There is, however, a considerable variation in the size of the egg-chambers of these flies, and a certain percentage of the flies develop their ovaries normally in spite of the allatectomy.* As the flies in the present experiment were only kept for 7 days after the operation it is difficult to estimate the percentage of flies with normally developing ovaries. In the older experiments, in which the flies were kept for 14 days after the operation, any degeneration of the egg-chambers was easily seen, and in that case it was found that 14% of the flies, allatectomized when less than 8 hr. old, developed their ovaries in spite of the removal of the corpus allatum. In the present experiments one of the flies had egg-chambers which measured 0·92 mm. (this one is not included in the calculation of the mean); in this case the yolk filled half of the egg-chamber. Six flies possessed egg-chambers measuring from 0·30 to 0·37 mm., the yolk filling from one-fifth to one-third of the egg-chambers; presumably these six flies would have developed their ovaries normally, if they had lived for some more days.

(2) *Effect on fat-body.* Besides its effect on the ovaries allatectomy also had a profound influence on the fat-body (see also E. Thomsen, 1942, p. 356) evidenced by a marked hypertrophy of the fat-body in the abdomen, as compared with normal females of the same age, which at that time generally possess ovaries with ripe eggs. The cells of the fat-body of allatectomized flies are larger than normal and their content of fat is abundant, making the cells look white. The great content of fat is easily demonstrated by staining pieces of the fat-body with Scharlach red. Generally the cells also contain a large amount of glycogen. The glycogen is easily demonstrated by putting a small piece of the fat-body into distilled water, in which the glycogen pours out of the cells as an opalescent fluid. (Pflüger's test was used to demonstrate that this substance was actually glycogen.)

(3) *Effect on accessory glands.* In the older experiments it was also found that the accessory glands of allatectomized females were smaller than those of normal, mature females. As castration had no effect on these glands, it was proved to be an effect of the corpus allatum. (For a description of the accessory glands of the female *Calliphora* see E. Thomsen, 1942, p. 366, Pl. XL, fig. 11). In the present experiments the two accessory glands were detached from the oviduct at both ends before they were measured, and accordingly the figures are greater than those given in my previous paper, in which the glands were only detached at the apex, otherwise remaining in connexion with the oviduct. (This implies that the figures are a little below the actual length of the glands, owing to the difficulty in observing the exact position of the end of the gland.) The lengths of the accessory glands of thirty-two flies were measured. In twenty-three individuals the length of both glands are given, in the remaining nine flies only one of the glands was measured. The lengths of the fifty-five accessory glands varied from 1·4 to 2·6 mm., the mean value being 1·91 mm. $\pm$ 0·06. The glands were rather flat and contained no secretion.

B. *Extirpation of the two medial groups of neurosecretory cells*

(1) *Technique*

Removal of the neurosecretory cells is only possible, because in the living fly under a binocular (64×) the cells look bluish white, which makes them distinguishable from the surrounding brain tissue. The neurosecretory cells resemble tiny rings, the transparent part in the centre being the nucleus. As a rule the cells are more conspicuous in the mature female than in the newly hatched female, the neurosecretory cells of which are lighter in colour, and consequently more difficult to distinguish.

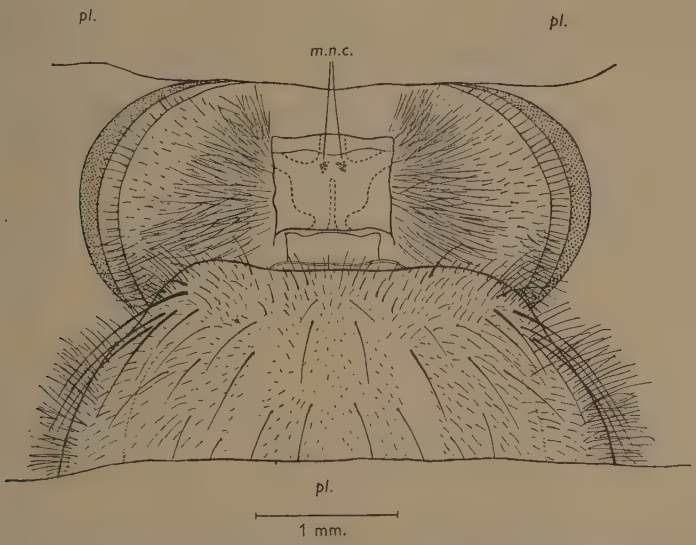


Fig. 3. Head of fly tilted forward exposing the posterior surface of the head showing the incision for the removal of the medial neurosecretory cells (m.n.c.). *pl.* plasticine keeping the fly in position.

The extirpation of the cells was performed in the following way: The fly was etherized and kept in position in the dissection dish with plasticine. The head was bent forwards to expose the posterior surface, which normally fits to the thorax (see Fig. 3). Two longitudinal incisions connected by a transverse cut were made centrally in the back of the head, and the small square lobe of the skin (cuticle + hypodermis), adhering at its posterior border to the head, was tipped backwards and fastened under the anterior margin of the thorax. (In Fig. 3 the small part of the skin is shown removed.) The next part of the operation was done under Ringer's fluid (composition: 7·5 g. NaCl + 0·35 g. KCl + 0·21 g. $CaCl_2$ per l. H_2O). The great tracheae of the head (dotted lines in Fig. 3) were removed, and the two medial groups of neurosecretory cells, now visible, were taken out with forceps, and put

into a small dish with Ringer's fluid in order to ascertain that the cells had in fact been removed. Of course it is necessary to remove a tiny piece of the surrounding brain tissue together with the neurosecretory cells. After the operation the fluid was removed, the small patch of the cuticle was slipped back into its natural position, the head was dried with filter-paper and the wound sealed by means of paraffin wax. With some practice the operation can be performed in 10 min. or even less. After the operation the fly was kept at 25° C. and fed on meat, sugar and water.

(2) *Experiments*

The series includes twenty-seven experiments comprising altogether 161 females which had the two medial groups of neurosecretory cells removed, and fifty-three females which were operated upon in the same way except that the neurosecretory cells were not removed; they are termed operated controls. The age of the flies at the time of the operation varied from 5 to 8 hr. Before the operation the flies did not get any food. After the operation the flies were kept for 7 days, being fed as described above. Then they were dissected and the biggest egg-chambers were drawn and measured. When fed a few hours before dissection the intestine was found to be filled with red meat juice. Of the 161 females, 96, i.e. 60%, died.

(*a*) *Effect on ovaries*. The egg-chambers of the surviving sixty-five females measured from 0·12–0·17 mm. in length as seen in Fig. 2 (lower curve). As the deposition of yolk does not begin until the egg-chamber reaches a length of about 0·25–0·30 mm., none of these egg-chambers contained any yolk. The size corresponds to the size of egg-chambers of about 3 days old (25° C.) normal females (see Table 1). Of the fifty-three operated controls fourteen died, the mortality thus being only 27%. Of the surviving thirty-nine controls three flies oviposited, twenty-five flies had ripe eggs, nine flies had egg-chambers which measured 0·28–0·43–0·60–0·85–1·08–1·13 and 1·24 mm., all egg-chambers containing normal yolk; two flies only, i.e. 5%, had egg-chambers which measured 0·17 and 0·18 mm. The difference between the developmental stage of the ovaries of the flies deprived of their m.n.c. and that of the ovaries of the operated controls is striking. In the first case 100% of the ovaries underwent only a slight development, i.e. the egg-chambers grew from about 0·07 mm., the length of the egg-chambers of 5–7 hr. old normal females (E. Thomsen, 1948*b*, p. 78, Fig. 5), to at the most 0·17 mm. Of the operated controls, however, 95% had either fully mature ovaries or ovaries in normal development, 5% only having small slightly developed ovaries.

To find out if the ovaries of flies without m.n.c. would develop further if the flies were kept for a longer time after the operation, a series of experiments was made, in which the flies were kept for 14 days after the operation. In this series thirty-six females aged 6–8 hr. at the time of operation had their m.n.c. removed. The mortality was very heavy, being 78%. The biggest egg-chambers of the surviving eight flies ranged from 0·15 to 0·17 mm., thus being of just the same size as those of the flies which were only kept for 7 days after the operation. As the mortality of flies kept for 14 days was so very high, and as the result was just the same as in flies kept for 7 days only, no more experiments of this kind were carried out.

At an earlier time during the investigation (E. Thomsen, 1948*a*), when the number of females deprived of m.n.c. and allatectomized females was still rather small, I was apt to think that the clear-cut effect on the ovaries was due to an inactivity of the corpus allatum only. A comparison of the size of the egg-chambers of the greater number of flies without m.n.c. now available with the egg-chambers of allatectomized females shows, however (Fig. 2), that the presumed inactivity of the corpus allatum cannot be the only cause of the inhibition of the growth of the ovaries. The length of the egg-chambers of flies deprived of their m.n.c. was found to be *surprisingly constant*, ranging only from 0·12 to 0·17 mm.; the variation in the length of the egg-chambers of allatectomized females was, however, much greater, only 27% of these were of the same size as the egg-chambers of the flies deprived of their m.n.c., the remaining 73% being bigger. *This interesting fact shows at least that the influence of the m.n.c. on ovarian development is not effected solely through the corpus allatum; the m.n.c. must affect some other physiological process vital to egg-development* (*see* p. 162).

(*b*) *Effect on fat-body*. The fat-bodies of females deprived of their m.n.c. generally differ from the fat-bodies of allatectomized females. As a rule the cells are smaller and contain much less fat than the fat-body cells of allatectomized females. Owing to the small content of fat the cells are in most cases transparent or semi-transparent. In many cases the fat-body of flies deprived of their m.n.c. looked somewhat bluish, and when put in distilled water much glycogen poured out of the fat-body cells.

In some of the flies deprived of m.n.c., which died a few days after the operation, the abdomen was found to be extremely distended, in many cases even to such a degree that the ovipositor was extruded. Dissection showed that the distension was due to the crop being greatly dilated with fluid.

(*c*) *Effect on accessory glands*. The accessory glands of thirty of the females deprived of m.n.c. were measured. In fourteen of the flies only one of the glands was measured, in the remaining sixteen flies the length of both glands is given. The length of the forty-six glands varied from 1·4 to 2·7 mm., the mean value being 1·85 mm. $\pm$ 0·05. The glands were flat and contained no secretion.

A comparison between the length of the accessory glands of females deprived of m.n.c. and that of allatectomized females shows that the glands are of about the same length, and so it is impossible to judge whether the m.n.c. have a direct effect on the growth of the accessory glands or whether they exert their influence on the glands via the corpus allatum only.

(*d*) *Effect on corpus allatum*. Regarding the problem of a possible activation of the corpus allatum by the m.n.c. it is interesting that the corpora allata of females deprived of m.n.c. were found to be definitely smaller than those of the operated controls. (For the technique of measuring see E. Thomsen, 1942, p. 334.) In the present experiment the corpus allatum of the living fly was measured. As the corpora allata were drawn at a linear magnification of $\times$ 90, the figures given below must be divided by 8100 to get the real values. In Fig. 4 (upper curve) the relative areas of twenty-five corpora allata of the operated controls are given, the mean value being 80 mm.^{2} $\pm$ 4·2. In the lower curve of the same figure the relative areas of the

corpora allata of twenty-six of the females deprived of m.n.c. are plotted, the mean value being 50 mm.$^2 \pm 2{\cdot}6$. As was to be expected the difference between the two groups was found to be statistically significant. This finding shows that the growth of the corpus allatum is affected by the m.n.c.

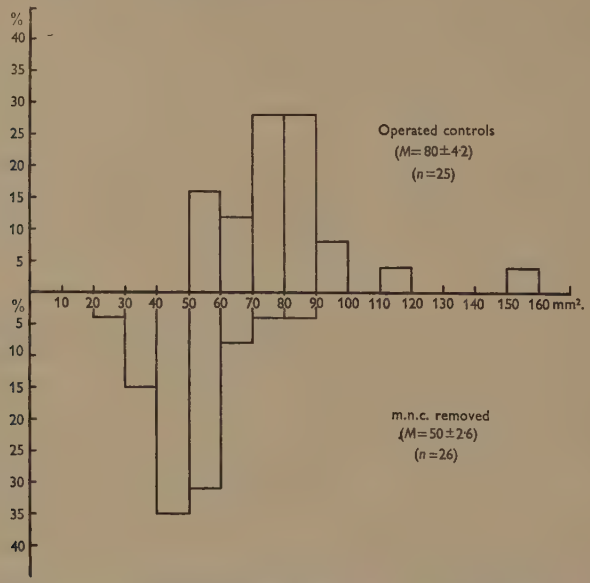


Fig. 4. Ordinate: percentage of flies. Abscissa: relative areas of corpora allata in mm.^{2} of operated controls and of females deprived of their medial neurosecretory cells (m.n.c. removed). Linear magnification × 90.

(3) *Extirpation of a piece of the brain without neurosecretory cells*

It might be objected that the effects of the removal of the m.n.c. were not due to the removal of the m.n.c. *per se*, but simply to the excision of a piece of the brain. To eliminate this possibility thirty-two females, 5–7 hr. old, had a piece of the brain behind the area containing the neurosecretory cells excised. Considered as controls these experiments are a bit dangerous, as the nerves from both the m.n.c. and the l.n.c. might be cut through. The lengths of the biggest egg-chambers of the surviving sixteen flies are shown in Table 2.

As only one fly had egg-chambers of the same length as the flies deprived of the m.n.c., it is clear that the inhibition of the development of the ovaries in the above-mentioned experiments was not due to the excision of a small piece of brain. The delayed development of the ovaries of some of the flies might be due to the severing of the nerves from the m.n.c. or the l.n.c. (see p. 151).

As a *summary* of the experiments described above we can state that the removal of the m.n.c. results in an inhibition of the growth of the ovaries. On an average the

Table 2. *Length of egg-chambers of flies deprived of a piece of brain, the neurosecretory cells being left in place*

0·13 mm.	Mature
0·25 mm.	Mature
0·25 mm.	Mature
0·37 mm.	Mature
0·72 mm.	Mature
0·83 mm.	Mature
0·83 mm.	Mature
0·97 mm.	Mature

development of the eggs ceases at an earlier stage than when the corpus allatum is removed, *and the size of the egg-chambers of the flies is extremely constant.* The fat-body contains less fat than the fat-body of allatectomized females, and the corpus allatum is smaller than those of the operated controls. The accessory glands were found to be of about the same length as the accessory glands of allatectomized females.

Although the experiments clearly show that the m.n.c. exert an influence on egg development, other than the probable regulation via the corpus allatum, it was decided to see if corpora allata from *mature* females were able to promote egg development in females deprived of their m.n.c. In previous experiments I have found that allatectomized females are able to produce ripe eggs when provided with corpora allata from mature females. The experiments will be described in the following section.

C. *Implantation of corpora allata from mature females into females deprived of their medial neurosecretory cells*

Females less than 8 hr. old were deprived of their m.n.c. The next day each of the surviving flies had three corpora allata from 7 or 8 days old females grafted into the abdomen by means of the transplantation method of Ephrussi & Beadle (1936). Owing to the slight turgor in the abdomen of the fly it is very difficult to penetrate the cuticle with the glass needle, even if it has got a fine point, and so a small incision was made in the abdomen by means of scissors. The incision was made on the left side of the abdomen in the hind part of the third abdominal segment. The wound was sealed with paraffin wax, and care was taken that the neighbouring spiracle was not closed. After the operation the flies were kept for 7 days before they were dissected, being fed in the usual way.

Of thirty-five females with implanted corpora allata seventeen survived. The lengths of the egg-chambers are given in Fig. 5. If the size of these egg-chambers is compared with the size of egg-chambers of flies deprived of their m.n.c. (Fig. 2, lower curve), it is seen that the implanted corpora allata in most cases induced some growth of the egg-chambers, but were unable to further the growth of the ovaries beyond the initial stage of yolk deposition. In earlier experiments (E. Thomsen, 1942, p. 358) it was found that corpora allata from mature females of *Calliphora* when grafted into allatectomized females caused after 7 days a normal development of the ovaries

of these flies in 93 % of the cases. In nearly all cases even mature eggs were produced. Thus the fact that in the present investigation the implanted corpora allata only caused a slight development of the ovaries cannot be due to an inactivity of the implanted corpora allata.*

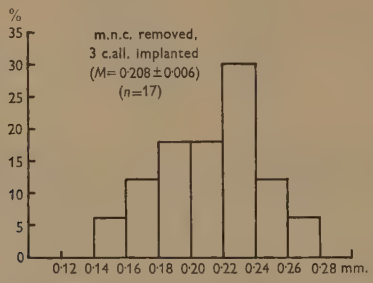


Fig. 5. Ordinate: percentage of flies. Abscissa: length of egg-chambers in millimetres of females deprived of their medial neurosecretory cells and provided with three corpora allata from mature females.

This result, that transplanted corpora allata from mature females promote a slight growth of the ovaries of females without m.n.c., indicates that the original corpora allata of the recipients might have been inactive. On the other hand, *the inability of the implanted corpora allata to produce a normal development of the ovaries confirms the theory of an influence of the m.n.c. on egg development in addition to the probable effect via the corpus allatum.* These experiments also show that the function of the corpus allatum of the normal fly is not confined to a regulation of the deposition of yolk in the oocyte, but that it must have an effect on the growth of the egg-chambers.

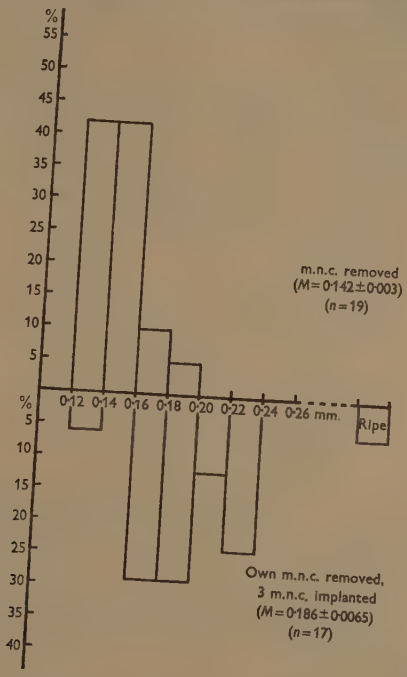
D. *Transplantation of medial neurosecretory cells from mature females into females deprived of their own medial neurosecretory cells*

If the effect of the m.n.c. on egg development is brought about by a hormone produced by these cells, an implantation of the m.n.c. from mature females into females deprived of their own m.n.c. should promote growth of the ovaries. Such experiments were therefore carried out.

Altogether 116 flies were deprived of their m.n.c. when less than 8 hr. old; twenty-nine of these died before the next operation, which was performed on the following day. Of the surviving flies thirty-five had three (double) groups of m.n.c. from 7–8 days old females implanted (for the technique of transplantation see p. 147), and the remaining fifty-two flies, serving as operated controls, received an injection of Ringer's fluid. Naturally it is impossible to implant the m.n.c. without a small piece of the surrounding brain tissue. After 7 days eighteen of the thirty-five flies

* In the older experiments the transplantation of the corpora allata was performed by means of forceps, in the present experiments by means of the transplantation method of Ephrussi & Beadle, but in other experiments mentioned on p. 153 I tried both methods of transplantation with the same result. So the divergent results cannot be due to the difference in technique.

with implanted m.n.c. and nineteen of the operated controls were alive. The length of the biggest egg-chambers of the two kinds of flies is given in Fig. 6. It is seen that the implanted neurosecretory cells in six cases, i.e. 36%, caused a development of the ovaries beyond the developmental stage of the controls; *in a single case, even normal ripe eggs measuring* 1·47 *mm. were produced.* The fact that the length of



g. 6. Ordinate: percentage of flies. Abscissa: length of egg-chambers in millimetres of females deprived of their medial neurosecretory cells (m.n.c. removed), and of females deprived of their own medial neurosecretory cells, but provided with three groups of medial neurosecretory cells from mature females (own m.n.c. removed, 3 m.n.c. implanted).

g-chambers of the great number of flies deprived of their m.n.c. in this investigan in no case exceeded 0·18 mm., shows, it seems to me, that the production of e eggs must be due to the implantation of the neurosecretory cells. The difference ween the size of the egg-chambers of flies with grafted neurosecretory cells and se of the operated controls was found to be statistically significant. (The fly with ure eggs was not included in the calculation of the mean value.)

urther, I tried to see if the implantation of a piece of brain tissue without rosecretory cells had any effect on ovarian growth. Nineteen females were rived of their m.n.c. when less than 8 hr. old, and into the abdomen of each was lanted a rather large piece of brain tissue (without neurosecretory cells) from a days old female. Fig. 7 shows that after 7 days the length of the egg-chambers of

these flies (with one exception) did not exceed the length of egg-chambers of flies only deprived of their m.n.c. In the exceptional case the egg-chamber measured 0·20 mm. As an egg-chamber of the same length was found in a female fed on sugar and water only (for an explanation see p. 159), it is impossible to decide whether the implanted piece of brain was responsible for the additional growth of this egg-chamber or not.

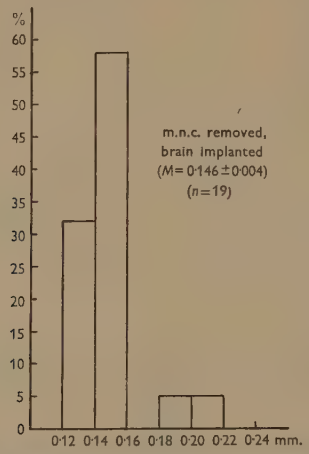


Fig. 7. Ordinate: percentage of flies. Abscissa: length of egg-chambers in millimetres of females deprived of their medial neurosecretory cells and provided with a piece of brain without neurosecretory cells.

It can therefore be concluded that the growth of the ovaries in the females deprived of their own m.n.c. but provided with m.n.c. from mature females is due to a hormone produced by the implanted m.n.c.

The fact that the implantation of m.n.c., with one exception, caused only a few of the ovaries to grow—and then only to a slight degree—might be explained by a reduced formation of hormone by the isolated cells. It is, however, not possible to decide whether the implanted m.n.c. act directly on the ovaries or whether they influence the corpus cardiacum-allatum system, which in turn stimulates the ovaries to grow (see p. 157.)

IV. THE FUNCTION OF THE LATERAL NEUROSECRETORY CELLS

Hitherto only the function of the two medial groups of neurosecretory cells has been investigated. As, however, Williams (1948*a*) found that in the pupa of *Platysamia cecropia* the medial and the lateral neurosecretory cells possibly co-operate in the regulation of the diapause, I tried to see whether the lateral groups of neurosecretory cells in *Calliphora* were indispensable or not for the development of the eggs.

Each lateral group of neurosecretory cells comprises three cells, two of which are situated close together, while the third cell lies a little apart (Fig. 1). As the l.n.c.

are not so easily seen, the removal of these cells is more difficult than the excision of the two medial groups, and very often the operation fails. Accordingly, the number of females successfully deprived of their two groups of l.n.c. is rather small. All in all, twenty-one females less than 8 hr. old had these cells removed. Of these flies fourteen were alive on the 7th day, when autopsy was performed. The lengths of the biggest egg-chambers are given in Table 3. It is seen that in four cases ripe eggs were produced, in three cases the egg-chambers measured 0·63, 0·72 and 0·73 mm., all containing normal yolk in the oocyte which filled about half of the egg-chamber. The remaining seven egg-chambers measured from 0·17 to 0·28 mm., and in the three biggest of these the yolk filled about one-quarter of the length. *This seems to show that the lateral neurosecretory cells have some influence on egg development, but the part played by these cells is of minor importance.* However, it cannot be entirely excluded that the effect is due to the mere excision of a piece of brain tissue. (See the experiments involving removal of a piece of brain, p. 147, Table 2.)

Table 3. *Length of egg-chambers of flies deprived of their two groups of lateral neurosecretory cells*

0·17 mm.	0·63 mm.
0·22 mm.	0·72 mm.
0·23 mm.	0·73 mm.
0·27 mm.	Mature
0·28 mm.	Mature
0·28 mm.	Mature
0·28 mm.	Mature

V. THE FUNCTION OF THE CORPUS CARDIACUM

A. *Removal of the ring-gland from young females followed by implantation of corpora allata from mature females*

The function of the corpus cardiacum of adult insects has hitherto received only little attention (see discussion, p. 165). It has, however, been proved beyond any doubt for several adult insects that the corpus cardiacum produces a hormone which influences the colour change of crustaceans (Brown & Meglitsch (1940), and independently of these authors Hanström (1940) and M. Thomsen (1943)). In their paper on neurosecretion B. & E. Scharrer (1944) suggest that the m.n.c. of the brain and the corpus cardiacum might be regarded as one neuro-endocrine complex, the role of which in the developing insect is the regulation of growth and differentiation. (At that time the existence of l.n.c. was unknown.) The fact that the corpus cardiacum of the adult insect produces a hormone, the effect of which in the physiology of the insect itself is still unknown and the position of the corpus cardiacum in relation to the neurosecretory cells and the corpus allatum, made the inclusion of the corpus cardiacum in the present study inevitable.

Now it must be remembered that the young flies, which had their m.n.c. removed, retained their corpus cardiacum; one might therefore think that in these experiments the function of the corpus cardiacum had been investigated and found to be nil with respect to its influence on egg development. But since the corpus cardiacum is

innervated from the neurosecretory cells of the brain, the possibility exists that a removal of these cells might render the corpus cardiacum inactive, and consequently the effect produced might be due to the inactivity of the corpus cardiacum.

In order to elucidate the functional significance of the corpus cardiacum two different kinds of experiments were performed; they will be described in the present and in the following sections.

In the first experiments I tried to see whether the ovaries were able to develop in females deprived of their corpus cardiacum. It must be borne in mind that the corpus cardiacum is a component of the ring-gland, a complex organ (see footnote on p. 141). Unfortunately, it is not possible to remove the corpus cardiacum without removing the side-lobes and the corpus allatum as well. Corpora allata from mature females were therefore implanted into females deprived of their whole ring-gland. In these experiments what was tested was whether the ovaries were able to develop in females possessing only the neurosecretory cells of the brain and the corpus allatum.

The removal of the ring-gland involves the removal of a small piece of the aorta, which is surrounded by the ring-gland. Fortunately, it was found that the cutting of the aorta had no effect on egg development. The operation also involves the severing of the two nervi oesophagi, arising from the fused corpus cardiacum and ganglion hypocerebrale and running to the anterior part of the gut (see E. Thomsen, 1942, p. 332, Text-fig. 1). Since the cutting of these nerves might be expected to cause a slowing down of digestion, resulting in a retardation in the development of the ovaries, initial experiments were made in which these nerves were cut.

Twenty-five females less than 8 hr. old had their aorta and nervi oesophagi cut through. The flies were kept for 7 days and fed as usual. In Table 4 it is seen that in most cases a definite retardation of the growth of the ovaries of the fourteen surviving flies was found. Furthermore, in the three cases where mature eggs were produced the number of eggs in each ovary was only about fifty, the normal number being about a hundred. Also the length of the eggs was smaller than usual, measuring 1·19, 1·26 and 1·33 mm. as compared with 1·44 mm., the average length of normal eggs.

Table 4. *Length of egg-chambers of flies in which the nervi oesophagi were cut*

0·15 mm.	0·37 mm.
0·17 mm.	0·58 mm.
0·23 mm.	0·84 mm.
0·25 mm.	1·04 mm.
0·27 mm.	1·19 (mature)
0·28 mm.	1·26 (mature)
0·28 mm.	1·33 (mature)

However, as the fat-body of these flies showed a high degree of hypertrophy, the fat-body cells being loaded with much fat and glycogen, the delay in egg development could hardly be due to an effect on the digestion and absorption of food. The effect must be due to a slowing down of the rate of mobilization of reserve substances

for egg production. As the corpus allatum of these flies was left completely intact this finding is difficult to understand.*

This finding, that the cutting of the nervi oesophagi causes a delay in egg development, must be borne in mind when interpreting the results of experiments in which these nerves are cut.

Table 5. *Length of egg-chambers of flies*

Ring-gland removed (mm.)	Ring-gland removed, 3 corpora allata implanted (mm.)	
0·08	0·12	
0·08	0·20	
0·10	0·23	(35 %)
0·10	0·25	
0·10	0·28	
0·12	0·28	
0·12	0·30	
0·12	0·32	
0·13	0·40	
0·13	0·40	
0·14	0·43	
0·15	0·45	65 %
0·15	0·50	
0·15	0·68	
0·15	1·04	
0·15	1·12 (mature)	
0·17	1·30 (mature)	
0·18	(n=17)	
0·19		
0·20		
0·28		
(n=21)		

After these initial control experiments forty-three females less than 8 hr. old were deprived of their ring-glands, and each was immediately provided with three corpora allata from 7–8 days old females. In some cases the transplantation of the corpora allata was performed by means of forceps, in other cases by means of the transplantation method of Ephrussi & Beadle, but as there was no difference in the results obtained all experiments are treated together. The operation was performed as described on p. 147. Forty-six females of the same age, deprived of their ring-gland and injected with Ringer's fluid, served as operated controls. All the flies were kept for 7 days after the operation and fed in the usual way. The length of the egg-chambers is given in Table 5. It is seen that there is great variation in length of the egg-chambers of the surviving twenty-one operated controls, but none exceeds 0·28 mm. The lengths of the egg-chambers of the surviving seventeen females with implanted corpora allata also showed great variation, but in 65 % of the cases they exceeded those of the operated controls and in two flies ripe eggs were produced. These eggs were, however, smaller than usual, measuring 1·12 and 1·30 mm. respectively. In these cases the number of eggs in each ovary was only about half

* I had the pleasure of discussing this problem with Dr Wigglesworth, who has permitted me to state that he suggested the possibility that the corpus allatum might be stimulated to release its hormone by afferent fibres in the nervi oesophagi. This problem certainly deserves a special investigation.

of the number generally found. In one fly all the egg-chambers had obviously degenerated, and in several cases some egg-chambers had degenerated, while others were quite normal.

The experiments show that flies are able to develop eggs in the absence of the corpus cardiacum. It was thus proved that the neurosecretory cells and the corpus allatum alone are able to further the development of the ovaries; but it is impossible to decide whether the retardation in the development of the ovaries is due to the severing of the nervi oesophagi or to the absence of the corpus cardiacum or to both causes.

It is noteworthy that the egg-chambers of flies deprived of the ring-gland and not provided with corpora allata in some cases were found to be smaller than egg-chambers of females deprived of their m.n.c. The great variation in the length of the egg-chambers might be due to a more or less pronounced residual effect of the removed organs. As a rule the fat-body was hypertrophied, and in many cases remnants of the larval fat-body persisted. The incomplete breakdown of the larval fat-body may be due to the removal of the side lobes of the ring-gland (see p. 163).

In some cases the flies deprived of their ring-glands exhibited a marked distension of the abdomen, and, just as was the case in flies deprived of m.n.c., the distension was due to the greatly dilated crop.

As these experiments did not elucidate the possible influence of the corpus cardiacum on egg development, the problem was tested in another series of experiments described in the next section.

B. *Extirpation of the medial neurosecretory cells followed by an implantation of the corpus cardiacum-allatum system from mature females*

If the corpus cardiacum has an influence on egg development similar to that of the m.n.c., and if it is activated by these cells, an implantation of corpora cardiaca from *mature* females into females deprived of their m.n.c. ought to promote a development of the ovaries. Tests were therefore made to see if transplanted corpora cardiaca from 7 days old females, i.e. presumably activated corpora cardiaca, had the supposed effect. As the corpus allatum of the host (without m.n.c.) is probably inactive, the corpus allatum was implanted together with the corpus cardiacum.

A number of females were deprived of their m.n.c. and kept as usual till the next day, when each of the flies had three corpus cardiacum-allatum systems from 7–8 days old females implanted. The corpus cardiacum-allatum system of such old females comprises the fused corpus cardiacum and ganglion hypocerebrale, the nervi allati and the corpus allatum, the side-lobes having completely disappeared on the fourth day (E. Thomsen, 1942, p. 336). As this system surrounds the aorta, a small piece of this vessel had to be implanted together with the ring-gland. Fifty-eight females had the m.n.c. removed, fifteen of these died or showed signs of weakness before the next operation, so only forty-three of the females had corpora cardiaca-allata systems implanted. Females deprived of their m.n.c., but provided with three corpora allata from mature females (see p. 147), were used as controls. Twenty of the flies deprived of m.n.c. and with three corpora cardiaca-allata systems implanted

were alive on the 7th day, when they were dissected. The lengths of the biggest egg-chambers are given in Table 6. It is seen that in eight of the cases, i.e. in 40%, the growth of the egg-chambers did not exceed that of egg-chambers of flies which were only provided with corpora allata, but in all the other cases, i.e. 60%, the size of the egg-chambers is definitely larger, in three of the cases even ripe eggs were produced. Furthermore, the egg-chambers of the developing ovaries looked quite normal, and in all cases but one the yolk filled from one-quarter to one-half of the egg-chamber. In the three flies with ripe ovaries the number of the eggs was normal, each ovary containing about eighty eggs and the length of the eggs was likewise normal, measuring 1·44, 1·47 and 1·47 mm.

Table 6. *Length of egg-chambers of flies*

m.n.c. removed, 3 c.all. implanted (mm.)	m.n.c. removed, 3 c.card. allata implanted (mm.)	
0·15	0·18	
0·17	0·18	
0·17	0·23	
0·18	0·23	
0·18	0·23	40%
0·19	0·23	
0·20	0·23	
0·20	0·27	
0·20	0·28	
0·22	0·32	
0·22	0·33	
0·23	0·33	
0·23	0·35	
0·23	0·38	
0·25	0·38	60%
0·25	0·48	
0·27	1·02	
(n=17)	Mature	
	Mature	
	Mature	
	(n=20)	

The striking difference in the size of the egg-chambers in females deprived of m.n.c. and with three corpora allata compared with females deprived of m.n.c. and with three corpora cardiaca-allata systems implanted permits the conclusion *that the corpus cardiacum from mature females may replace the m.n.c., having a similar effect on egg development as these cells.* The delay in the development of the eggs found in all cases but three is easily explained; in the normal fly the growth of the ovaries is governed both by the m.n.c. and by the corpus cardiacum, but in this case only by the implanted corpora cardiaca. The influence of the corpus cardiacum on egg development is clearly *brought about by a hormone released by this organ.*

Furthermore, the experiments show beyond any doubt that the corpus cardiacum must be activated by the m.n.c. in order to accomplish its function. From this fact it is inferred that the effects produced by a removal of the m.n.c. were due primarily to the lack of these cells and secondarily to the non-functioning of the corpus cardiacum.

VI. THE ACTIVATION OF THE CORPUS CARDIACUM-ALLATUM SYSTEM

As to the way in which the m.n.c. (and to a minor degree probably the l.n.c.) regulate the activity of the corpus cardiacum-allatum system three possibilities exist. The first possibility, is that the corpus cardiacum-allatum system is stimulated by a nervous impulse. Another possibility is that the activity of the corpus cardiacum-allatum system is regulated by a hormone produced by the neurosecretory cells, and finally the two methods of regulation may be combined.

To test these possibilities, the following experiments were made. From females less than 8 hr. old the ring-gland was removed and immediately grafted into the abdomen of the same fly. In this way the nervous connexion between the neurosecretory cells and the corpus cardiacum-allatum system was broken in a rather drastic way. Out of sixty-one flies which had their ring-gland transplanted into the

Table 7. *Length of egg-chambers of flies*

Ring-gland removed (mm.)	Ring-gland removed and re-implanted (mm.)	
0·08	0·12	
0·08	0·18	
0·10	0·18	
0·10	0·18	
0·10	0·19	50%
0·12	0·19	
0·12	0·21	
0·12	0·23	
0·13	0·28	
0·13	0·28	
0·14	0·32	
0·15	0·35	
0·15	0·38	
0·15	0·39	
0·15	0·45	50%
0·15	0·53	
0·17	0·68	
0·18	0·83	
0·19	1·26 (mature)	
0·20	1·33 (mature)	
0·28	(n=20)	
(n=21)		

abdomen, twenty flies were alive on the 7th day, when autopsy took place. Flies which had their ring-gland removed and which received an injection of Ringer's fluid (p. 153) were used as controls. The length of the egg-chambers of the flies is given in Table 7. It is seen that the egg-chambers of the controls measured from 0·08 to 0·28 mm. Ten of the flies with transplanted ring-glands, i.e. 50%, had egg-chambers which measured from 0·12 to 0·28 mm., and eight flies had egg-chambers the lengths of which varied from 0·32 to 0·83 mm., containing normal yolk occupying one-fourth to half of the length of the egg-chamber. Some of the egg-chambers of the ovaries were obviously degenerate, whereas others were quite normal. Finally,

two of the flies possessed ovaries with ripe eggs: these eggs measured, however, only 1·26 and 1·33 mm.

It is seen that in 50% of the cases the transplanted ring-gland was able to promote a development of the ovaries beyond the stage of the controls, and that in two cases even fully ripe eggs were produced.

The experiments clearly show that the corpus cardiacum-allatum system is activated at least to a certain degree by a hormone produced by the m.n.c. and that this hormone can be transmitted via the blood. The reason why the ovaries of 50% of the flies did not respond to the implanted ring-gland may be that normally the corpus cardiacum-allatum system is activated both by nervous and by humoral means, but it might equally well be due to the severing of the nervi oesophagi (see p. 152). In the last chapter it was shown that the corpus cardiacum is stimulated by the m.n.c. Whether the m.n.c. have a direct effect on the corpus allatum is impossible to say. But as the corpus allatum of normal flies is innervated via the corpus cardiacum it is most probable that normally the corpus allatum is activated via the corpus cardiacum and not directly by the m.n.c.

As the cytology of the neurosecretory cells has not yet been investigated, it is impossible for the time being to say whether a transport of secretion along the axons from the neurosecretory cells to the corpus cardiacum-allatum system may play a part in the activation of this system. B. & E. Scharrer (1944) describe granules of secretion along the axons from the neurosecretory cells to the corpus cardiacum of *Leucophaea maderae*.

On the basis of the experiments described above the function of the endocrine system of the adult *Calliphora* female is interpreted as follows:

The medial neurosecretory cells must be regarded as the overall controlling centre of the endocrine system. Probably these cells *per se* have a hormonal influence on egg development; besides this function they activate the corpus cardiacum, which has a similar influence on egg growth as the m.n.c. The corpus allatum, which was formerly found to stimulate ovarian development and the metabolism, is in all probability activated by the m.n.c. via the corpus cardiacum. The activation of the corpus cardiacum-allatum system by the m.n.c. is at least partly effected by means of a hormone given off by these cells. The l.n.c. probably collaborate with the m.n.c. in their function but are of minor importance.

The results of these experiments, showing that the neurosecretory cells and the corpus cardiacum have a similar function, corroborate the interpretation of B. & E. Scharrer (1944) that the neurosecretory cells of the brain and the corpus cardiacum of the developing insect are to be regarded as components of one neuroendocrine system.

VII. PHYSIOLOGICAL SIGNIFICANCE OF THE MEDIAL NEUROSECRETORY CELLS AND THE CORPUS CARDIACUM

But how is the influence of the neurosecretory cells and the corpus cardiacum on egg development brought about? I should like to *emphasize* that in the above-mentioned experiments the ovaries have only been used as indicators for the function

or non-function of the investigated organs. The neurosecretory cells and the corpus cardiacum might have a specific influence on the ovaries alone. Since, however, the growth of both the corpus allatum and the accessory glands of females deprived of m.n.c. was also arrested, this seems unlikely.

The simultaneous occurrence of small ovaries and a scanty content of fat in the fat-body cells of most of the females without m.n.c. is also noteworthy, as in 'normal' (i.e. non-operated) females the retardation in the development of the ovaries, which sometimes occurs from unknown causes, is generally followed by a marked hypertrophy of the fat-body owing to a great content of fat in the fat-body cells.

These facts suggest that the influence on the ovaries is not a specific one, and it seems possible that the m.n.c. and the corpus cardiacum in some way or other exert a profound influence on metabolism.

It seems to me that the problem of the function of the medial neurosecretory cells and the corpus cardiacum in the adult Calliphora *may be regarded as a problem of growth.* Even if the adult fly does not grow any more, some of its organs grow; this applies to the ovaries, the fat-body, the corpus allatum and the accessory glands. I have found it impossible to measure the growth of the fat-body. As to the growth of the other organs, the growth of the ovaries is by far the most conspicuous. This is seen from the following. The egg-chambers of twenty-one females 5–7 hr. old measured on an average 0·07 mm.; as the average length of a ripe egg was 1·44 mm. (120 eggs from twenty individuals were measured) it is seen that the linear growth of an egg-chamber amounts to about 21 × the initial length. When one remembers that only the linear growth was measured, no regard being paid to the volume, it is indeed remarkable growth, generally performed in 7 days or even less.

The growth of the corpus allatum of the adult fly has previously been investigated by the author (E. Thomsen, 1942, p. 334). It was found that the growth of the corpus allatum (calculated on the area) amounted to 3·7 × the initial area.

The mean value of twenty-eight accessory glands from seventeen newly emerged females was 1·56 mm. ± 0·05. As the mean value of thirty-five accessory glands from eighteen 7 days old females was 2·95 mm. ± 0·06, it is seen that the accessory glands nearly double their length.

As growth means that synthesis of protein takes place, the fact that the growth of the egg-chambers, the corpus allatum and the accessory glands of flies deprived of their m.n.c. was restricted *suggests that the metabolism of protein is abnormal when the m.n.c. are lacking.*

Looking at the results from this point of view it was of interest to see how far the ovaries, the corpus allatum and the accessory glands are able to grow in flies fed on a diet not including meat, i.e. on sugar and water. It has been stated several times (Evans, 1935; Fraenkel, 1940; Trager, 1947; Wigglesworth, 1950) that blow-flies thrive well on such a diet, but that they cannot produce mature eggs unless they are fed protein. But as far as I know there has been no investigation of the stage to which the development of eggs will proceed in the absence of protein, at least I have never seen any record of the length of egg-chambers of *Calliphora* fed sugar and water.

This also applies to the size of the corpus allatum and the accessory glands of such flies. Such an investigation was therefore undertaken.

Thirty-eight flies were kept for 7 days in the usual way and fed sugar and water only; the lengths of the egg-chambers and of the accessory glands and the area of the corpora allata were measured. (In what follows these flies will be called 'sugar flies'.)

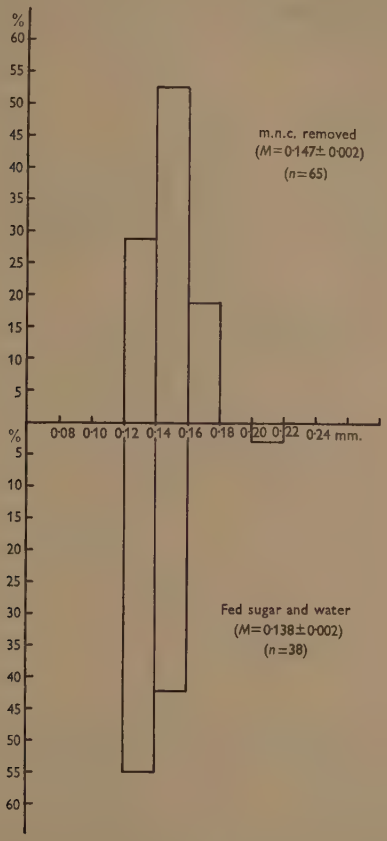


Fig. 8. Ordinate: percentage of flies. Abscissa: length of egg-chambers in millimetres of females deprived of their medial neurosecretory cells (m.n.c. removed) and of females fed sugar and water.

(1) *Egg-chambers.* If the lengths of the egg-chambers of the 'sugar flies' (Fig. 8, lower curve) are compared with those of flies a few hours old (p. 158) it is seen that the egg-chambers on an average doubled their length. Furthermore, it is remarkable (Fig. 8) that the egg-chambers of 'sugar flies' are of very nearly the same size as

those of females deprived of m.n.c., the mean value of the 'sugar flies' being 0·138 mm. ± 0·002 as compared with a mean value of 0·147 mm. ± 0·002 for the flies deprived of m.n.c.

(2) *The corpus allatum.* The mean of the areas of the corpora allata of the 'sugar flies' (relative figures) was 38 mm.^{2} ± 1·4 (Fig. 9, lower curve) as compared with 80 mm.^{2} ± 4·2 of females of the same age fed sugar, water *and* meat (see Fig. 4, upper curve), and so it is obvious that the full growth of the corpus allatum is also dependent on the intake of protein.

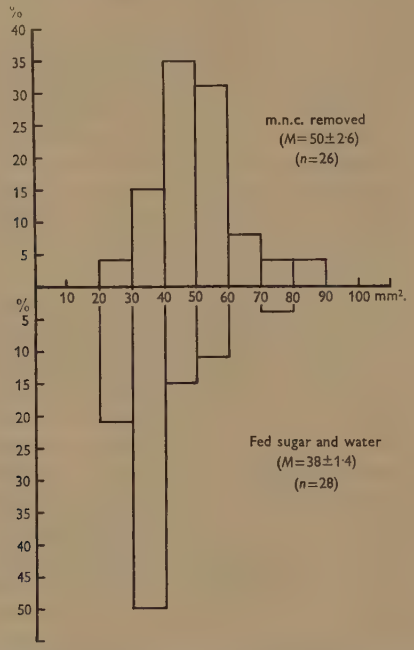


Fig. 9. Ordinate: percentage of flies. Abscissa: relative areas of corpora allata in mm.^{2} of females deprived of their medial neurosecretory cells (m.n.c. removed) and of females fed sugar and water. Linear magnification × 90.

Since the corpora allata of the sugar flies were measured in the living insect it is impossible to compare the corpora allata of these flies with the corpora allata of newly emerged flies, because in these the corpus allatum is imbedded in the side lobes of the ring-gland, and for this reason it is impossible to measure it exactly in the living fly. Consequently I am not able to state just how much the corpora allata grow in the 'sugar flies'.

The mean value of the corpora allata from females deprived of m.n.c. was 50 mm.^{2} ± 2·6 (Fig. 9, upper curve). As the difference between the means of the corpora allata of 'sugar flies' and of females deprived of m.n.c. was 4·73 × the error on the

difference, the difference is statistically significant. However, the corpus allatum of females deprived of m.n.c. were on an average 12 mm.^{2} larger than those of 'sugar flies' only, but they were 30 mm.^{2} smaller than the corpora allata of 7 days old females.

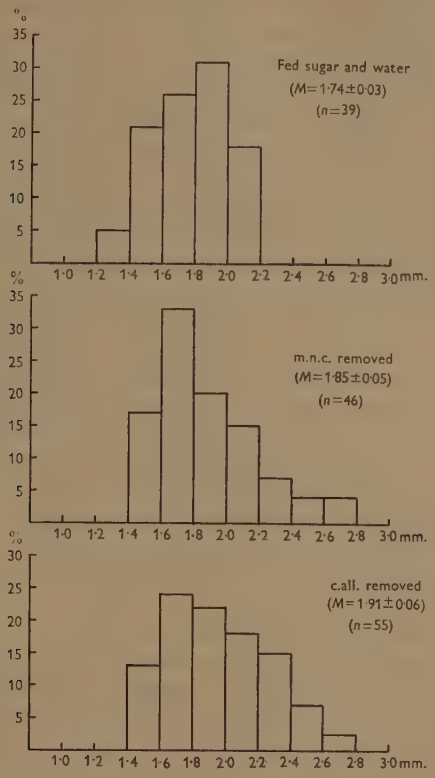


Fig. 10. Ordinates: percentage of accessory glands. Abscissae: length of accessory glands in millimetres, of females fed sugar and water, of females deprived of their medial neurosecretory cells (m.n.c. removed) and of allatectomized females (c. all. removed).

(3) *Accessory glands.* The mean length of thirty-nine accessory glands from thirty-two of the 'sugar flies' was 1·74 mm. ± 0·03 (see Fig. 10, upper curve). As the average length of the accessory glands of newly emerged females was 1·56 mm. ± 0·05, it is clear that even on a diet of sugar and water the accessory glands show some growth, but they do not nearly reach the length of the accessory glands of 7 days old females fed *meat*, sugar and water, which have an average length of 2·95 mm. ± 0·06.

The length of the accessory glands of females deprived of m.n.c. (Fig. 10, central curve) was approximately the same as that of 'sugar flies', the means being 1·85 mm. ± 0·05 and 1·74 mm. ± 0·03. The mean value of the accessory glands of allatecto-

mized females was somewhat larger (1·91 mm. ± 0·06; cp. Fig. 10, lower curve), but in reality the difference between this figure and the mean for the 'sugar flies' is only 2·54 × the error on the difference and not statistically significant. One therefore cannot decide with absolute certainty whether the growth of the accessory glands is regulated directly by the m.n.c. or via the corpus allatum. The difficulty is that the growth of the accessory glands is rather small.

The growth of the ovaries, the corpus allatum, and the accessory glands of 'sugar flies' quite certainly takes place at the expense of the protein of the larval fat-body, which is abundant in the newly emerged adult fly, but is broken down during the first days of adult life, and as a rule has disappeared on the third or fourth day.

Since the ovaries, the corpora allata and the accessory glands of flies deprived of m.n.c. are of nearly the same size as those of the 'sugar flies', it is reasonable to assume *that the growth of the organs of flies deprived of m.n.c. takes place mainly at the expense of the protein content of the larval fat-body, and that they are only able to use very little of the protein of the ingested meat.*

The small additional growth of the organs of the females deprived of m.n.c. as compared with the organs of the 'sugar flies' might be due to a slight activity of the l.n.c., or of the corpus cardiacum when stimulated by these cells, or to a residual effect of the removed m.n.c. The fact that the corpus allatum of flies deprived of m.n.c. grew comparatively more than the ovaries and the accessory glands of the same flies, is in favour of the hypothesis of a slight activity of the corpus cardiacum, since the corpus allatum is so closely connected with the corpus cardiacum.

Now it is evident that the above-mentioned experiments do not allow any conclusions to be drawn as to how the postulated effect of the m.n.c. and the corpus cardiacum on the protein metabolism is brought about. However, the influence must be hormonal.

It might be that the operated flies eat less than normal flies, or that the ingested food is not properly digested, or that the digested food is not properly absorbed, or that the building-up, the resynthesis, of the absorbed amino-acids is inhibited. The amazingly constant size of the egg-chambers of flies without m.n.c. is in favour of the suggestion that the synthesis is blocked. If so, the high content of glycogen in the fat-body of females deprived of m.n.c. might be explained by assuming that the amino-acids which presumably cannot be converted into protein are instead converted into glycogen. According to Wigglesworth (1942, 1950) protein may be converted into glycogen in insects.

On the other hand Fraenkel (1940) found that the adult *Calliphora* is not able to live for a longer time on meat and water than on water alone. Fraenkel suggests 'that the adult fly either cannot produce intermediary carbohydrates from protein, or does so at such a slow rate that it cannot maintain the ordinary muscle metabolism'. Presumably this means that the fly burns itself up, owing to the 'specific dynamic action' of the protein. When the fly is given sugar in addition to the meat as in my experiments it is conceivable that the protein could be converted into glycogen.

But assuming that the fly deprived of m.n.c. is unable to use the ingested protein for the growth of its organs, the problem arises of why the fly is capable of utilizing

the protein content of the larval fat-body. The anomaly might be explained if some factor which renders the fly capable of utilizing the protein content of the larval fat-body would disappear when the larval fat-body was broken down, and another system then took over the utilization of the ingested protein. Now it has been found that the side lobes of the ring-gland only persist during the first days of adult life, having completely disappeared on the fourth day. At that time the larval fat-body has also been broken down completely; so it might be that the side lobes are responsible for the breaking down of the larval fat-body and for the initial growth of the organs. When the side lobes have disappeared the utilization of the ingested protein might be taken over by the m.n.c. and the corpus cardiacum, and to a minor degree by the corpus allatum.

In this connexion it is of interest that the size of the egg-chambers of flies deprived of their m.n.c. corresponds to the size of normal egg-chambers on the third day (see Table 1, p. 140). It is also noteworthy that remnants of the larval fat-body were found only in 7 days old females when the operation included the removal of the side lobes of the ring-gland, and furthermore the egg-chambers of some of these flies were even smaller than the egg-chambers of flies deprived of their m.n.c. (see Table 5, p. 153). A similar persistence of some of the larval fat-body was also noticed by Day (1943) in *Lucilia* and *Sarcophaga* after the removal of the ring-gland.

As it has been found that the side lobes of the ring-gland in *Drosophila* (Vogt, 1943*a*) and in *Calliphora* (Possompès, 1950*a*) regulate the growth and differentiation of the imaginal disks, it is not unlikely that the side lobes of the ring-gland in the adult *Calliphora* are responsible for the breakdown of the larval fat-body, and the initial growth of the organs. It might also be that the protein of the larval fat-body is species-specific protein and therefore more readily usable than the ingested protein.

I should like to *emphasize* that the interpretation of the physiological significance of the m.n.c. and of the corpus cardiacum set forth in this section is only meant as a *working hypothesis*; further investigation may perhaps show it to be right or it may prove to be entirely wrong.

VIII. DISCUSSION

(1) *The neurosecretory cells.* The function of the neurosecretory cells in the brain of the *adult* insect has not hitherto been studied. But even if no special regard has been paid to the function of these cells in the adult, Wigglesworth (1936, p. 108) in his classical work on *Rhodnius* made some experiments to see if the brain as a whole was involved in the regulation of the development of the ovaries. In this insect an excision of the brain did not inhibit egg development. This result is in conflict with my finding in *Calliphora*, but it must be remembered that *Rhodnius*, though deprived of the brain, retained its corpus cardiacum intact, and so it might be that the corpus cardiacum had been activated by the neurosecretory cells before the latter were excised, and was thus able to take over the function of the neurosecretory cells.

B. Scharrer (1946*a*, p. 48) briefly states that in *Leucophaea* the removal of the brain suppressed the ovaries at least in a certain percentage of experimental animals.

In some insects it has been established that the brain has a definite effect on pupation. This effect of the brain is most certainly due to the neurosecretory cells. The literature on the hormones regulating the development of insects is by now extensive, and as an excellent review of the investigations dealing with this problem and a valuable discussion of the results obtained has recently been published by B. Scharrer (1948), I shall confine myself to discussion of the papers in which a special regard is paid to the neurosecretory cells.

The first investigation of the function of the neurosecretory cells of the brain was undertaken by Wigglesworth (1939, 1940) in *Rhodnius prolixus*. Wigglesworth showed that a factor regulating moulting is produced by the pars intercerebralis of the protocerebrum, in which part of the brain the neurosecretory cells are situated (Hanström, 1938). If a piece of this part of the brain containing the neurosecretory cells from fourth-stage nymphs at 6 or 7 days after feeding, or from fifth-stage nymphs at about 10 days after feeding was implanted into fourth-stage nymphs decapitated at 24 hr. after feeding, moulting was induced in 66% of the latter. Other parts of the brain had no such effect. These experiments show that the factor which induces moulting is produced by the neurosecretory cells. The implantation of the corpus cardiacum ('the sympathetic ganglion') caused no moulting.

A very thorough study of the function of the neurosecretory cells in the development of the moth *Platysamia cecropia* was made by Williams (1946, 1947, 1948*a*, *b*). In a series of brilliant experiments Williams showed that the diapause in the pupa of *Platysamia* is regulated by the brain, and that the active factor is produced by the medial and lateral groups of neurosecretory cells presumably working together. Besides the brain the prothoracic glands, activated by the brain, play a part in the regulation of the diapause. How the neurosecretory cells and the prothoracic glands act has not been fully elucidated, but there seems to be a correlation between the developmental factors and the cytochrome system of the tissue cells.

It seems logical to assume that the function of the neurosecretory cells of the adult insect should be similar to the function of these cells during development. The fact that these cells in both *Rhodnius* and *Platysamia* produce a growth-promoting factor during the development supports my suggestion that the neurosecretory cells of the adult *Calliphora* stimulate growth. Because the adult insect as a whole does not grow any more, one has been accustomed to think of growth in insects in connexion with the developmental stages only, therefore the hypothesis that the neurosecretory cells of the adult *Calliphora* should be concerned with growth may sound like a paradox. However, in many adult insects some organs do grow; in most cases this applies to the ovaries and the fat-body, and in the case of *Calliphora* also to the corpus allatum and the accessory glands.

When the growth of these organs is arrested as a result of the excision of the m.n.c., the organs could be said to enter into a diapause. Seen from this point of view the investigations of the m.n.c. described in this paper are naturally brought into line with the studies of Wigglesworth and those of Williams.

Dupont-Raabe (1949) has shown that in the larva of *Corethra* the brain and the corpus cardiacum produce a hormone which is responsible for the expansion of the

chromatophores. The fact that the brain and the corpus cardiacum in this insect have a similar effect supports the finding of the author that in *Calliphora* the m.n.c. and the corpus cardiacum influence the same physiological process.

Quite recently Rehm (1950) demonstrated that in *Ephestia kühniella* there is a correlation between the cytological signs of secretory activity of the medial neurosecretory cells and the so-called critical period, in which the brain secretes the factor inducing pupation ('Verpuppungshormon', Kühn & Piepho, 1936; Piepho, 1942). A similar correlation was found during the larval moults.

It must also be mentioned that Possompès (1950*b*) has shown that in the larva of *Calliphora erythrocephala*, the side lobes of the ring-gland ('la glande péritrachéenne') is activated by the brain. Possompès suggests that this activation might be due to the neurosecretory cells of the brain.

(2) *The corpus cardiacum.* The function of the corpus cardiacum of the adult insect has received but little attention. The first indication of the function of the corpus cardiacum during the development was given by Pfeiffer (1939, p. 452), who recorded that in *Melanoplus differentialis* removal of the corpus cardiacum resulted in a delay in moulting. This finding indicates that the growth of the grasshopper is influenced by the corpus cardiacum, and thus it supports my interpretation of the function of the corpus cardiatum of the adult female *Calliphora*.

As mentioned on p. 151, the corpus cardiacum of different adult insects was found to produce a hormone which influences the colour change of crustaceans (Brown & Meglitsch, 1940; Hanström, 1940; M. Thomsen, 1943), but the part played by the corpus cardiacum in the physiology of the adult insect itself remained obscure.

The first evidence of the function of the corpus cardiacum in an adult insect was given by Joly (1945) in *Dytiscus*. Joly found that cardiacectomy had the same effect on the ovaries as allatectomy, but since the removal of the corpora cardiaca was followed by a degeneration of the corpora allata, he ascribed the effect on the ovaries to the degeneration of the corpora allata (see also p. 166).

Vogt (1946) found that an implantation of corpora cardiaca from 4 days old adult female *Drosophila hydei* into young larvae (2 days and 21 hr. old) caused a delay in the formation of the puparium, whereas no such effect was found when corpora cardiaca were implanted into older larvae (no age given). These facts might suggest that it is the feeding period, i.e. the period in which the larva grows, which is prolonged. The corpus cardiacum was also found to have an influence on the colour of the puparium.

Cardiacectomy combined with allatectomy including the removal of the side lobes of the ring-gland has been performed by Day (1943) in the adult *Lucilia* and *Sarcophaga*, by Vogt (1943*b*, 1947) in *Drosophila*, and by the author in *Calliphora* (E. Thomsen, 1948*b*). The effect on the ovaries, the fat-body and the oenocytes found in the experiments on *Lucilia*, *Sarcophaga* and *Drosophila* is in all probability due not only to the allatectomy, but also to the excision of the side lobes of the ring-gland. Since I have now found in *Calliphora* that the m.n.c. of the brain and the corpus cardiacum have a similar function it is evident that removal of the corpus cardiacum alone cannot give conclusive information about the function of this organ.

Consequently in my experiments on *Calliphora* carried out in 1948, the effect on the ovaries of the removal of the whole ring-gland as compared with that of the extirpation of the corpus allatum alone is probably due to the excision of the side lobes of the ring-gland and not to the removal of the corpus cardiacum.

(3) *The corpus allatum.* During later years the function of the corpus allatum of the adult insect has been intensively studied (for a review of the literature see Joly (1948) and E. Thomsen (1948*b*); cp. also Bodenstein (1947)). It is generally agreed that the corpus allatum is necessary for the normal development of the ovaries; and in *Calliphora* the corpus allatum was also found to have a decisive influence on the growth of the accessory glands both in the female and in the male.

Furthermore, it has been proved beyond any doubt that in *Melanoplus differentialis* (Pfeiffer, 1945) and in *Calliphora erythrocephala* (E. Thomsen, 1949) the corpus allatum influences metabolism; the influence of the corpus allatum on the ovaries is most probably due to its metabolic effect. In both *Melanoplus* and *Calliphora* allatectomy results in a marked hypertrophy of the fat-body, the fat-body cells being loaded with fat. A similar hypertrophy of the fat-body was found after removal of the ring-gland in *Lucilia sericata* and *Sarcophaga securifera* (Day, 1943) and in *Drosophila hydei* (Vogt, 1947). In these insects an influence on the oenocytes was also reported, evidenced in the decrease in size of both the nucleus and the cytoplasm. As allatectomy in females of *Calliphora* was found to lower the oxygen consumption by 24%, the great accumulation of fat in the fat-body cells is probably due to reduced metabolism. So perhaps one of the functions of the corpus allatum of the adult female is to mobilize the material vital to the growth of the ovaries, as first suggested by Pfeiffer (1945) in her comprehensive paper.

In the present paper a definite influence of the corpus allatum on the initial growth phase of the ovaries was demonstrated (p. 147). As the corpus allatum also influences the growth of the accessory glands in both sexes, it is evident that the corpus allatum produces a growth-promoting factor. This finding corroborates the interpretation of the function of the corpus allatum in *Dytiscus* and *Carabus* set forth by Joly (1945, p. 131, 1948, 1950).

Studies performed during the last years (Pflugfelder, 1940; Pfeiffer, 1945; Vogt, 1943*b*; Wigglesworth, 1947, 1948) indicate that the juvenile hormone produced by the corpus allatum of the larva is identical with the corpus allatum hormone of the adult.

Concerning the problem of the activation of the corpus allatum very little is known. Wigglesworth (1934, 1940) was of the opinion that in *Rhodnius* the larval corpus allatum required to be stimulated by the moulting hormone in order to produce the juvenile hormone (see also Pfeiffer, 1945, p. 222). But later (Wigglesworth, 1948) he suggested that the normal functioning of the corpus allatum is only possible when the connexion between the central nervous system and the corpus allatum complex is intact.

This assumption is also favoured by Joly (1945, p. 146), who suggests that the corpus allatum is activated by a neuro-hormonal stimulation via the corpus cardiacum. This idea is in accordance with my assumption that in *Calliphora* the neurosecretory

cells stimulate the corpus allatum via the corpus cardiacum. On the other hand, I have found (p. 157) that the corpus allatum can be activated even when the nervous connexion between the brain and the corpus cardiacum is broken. This is in agreement with the finding of B. Scharrer (1946*b*) who recorded that in *Leucophaea* the cutting of the nerve corporis cardiaci does not prevent egg development.

However my result is in disagreement with that of Day (1943) who stated that in the adult female of *Lucilia* and *Sarcophaga* the severing of the recurrent nerve (which involves a section of the nervi interni and externi) has the same effect as extirpation of the ring-gland. Day also writes that the cutting of the nerve results in hypertrophy of the corpus allatum.

In the discussion on p. 157 I have suggested that in *Calliphora* the corpus cardiacum-allatum system is stimulated via both the nerve and the hormone by the m.n.c. of the brain.

The inhibition of the growth of the corpus allatum in females deprived of their m.n.c., interpreted as being due to the effect of these cells on protein metabolism, renders it difficult to decide if the corpus allatum is specifically stimulated by the m.n.c. Probably both the m.n.c. and the corpus cardiacum are concerned in the regulation of the formation of the corpus allatum hormone, the release of the hormone being perhaps due to other stimuli (see footnote on p. 153).

IX. FINAL REMARKS

Many biologists are still sceptical as to whether the inclusions in the neurosecretory cells are really secretory products. But after studying the long series of thorough cytological investigations of these cells in animals belonging to different systematic groups, mainly performed by E. & B. Scharrer and their collaborators, and by Hanström (see also M. Thomsen, 1951), I think that the view that the inclusions in the cells are artifacts must be abandoned.

As also pointed out by Hanström (1947*a*, *b*) the idea of a secretory activity of nerve cells is well in line with the finding of Parker (1932, 1936, 1948) that in fishes the nerve-endings produce substances, so-called neurohumours, which activate the chromatophores. The production of acetylcholin and sympathin by nerve-endings is also mentioned in this connexion.

The fact that the neurosecretory cells are widely distributed in the animal kingdom suggests that they may be concerned in the regulation of fundamental physiological processes. Since it has been shown that the neurosecretory cells of the brain in insects have a definite influence on such a fundamental physiological process as growth, it seems to me that this finding can be used as a starting-point for investigations on the neurosecretory cells of animals belonging to other systematic groups.

In this connexion I should like to call attention to the very illuminating analogy pointed out by Hanström (1941, 1947*b*) and by the Scharrers (1944), between the intercerebralis-cardiacum-allatum system of insects and the hypothalamo-hypophyseal system of the vertebrates. Remembering that the nucleus supraopticus and paraventricularis of the hypothalamus and the posterior pituitary gland are now

regarded as a functional unit, it is interesting that this also applies to the m.n.c. of the brain and the corpus cardiacum of the adult *Calliphora*. Considering the extensive literature of the hypothalamus and its connexion with the hypophysis, it is curious that until recently the description of neurosecretory cells in the hypothalamus has received so little attention.

The study of the functional significance of the neurosecretory cells is just at the beginning; further investigations on this problem are very much needed and will undoubtedly yield interesting and far-reaching results.

X. SUMMARY

1. The neurosecretory cells of the protocerebrum of the adult female *Calliphora erythrocephala* comprise two medial groups, each consisting of about eight cells, and two lateral groups, each consisting of three cells.

2. Since the corpus allatum is probably innervated from the neurosecretory cells of the brain, it was originally supposed that the function of the neurosecretory cells was the activation of the corpus allatum. Experiments were therefore carried out to discover whether extirpation of the neurosecretory cells had an effect like that of allatectomy.

3. The excision of the two medial groups of neurosecretory cells (m.n.c.) had an effect on the ovaries which was clearly different from that of allatectomy. From this fact it is inferred that the effect of the m.n.c. on ovarian development is not solely through the corpus allatum, but that the m.n.c. must affect some other physiological process vital to egg development.

4. The fat-body of females deprived of m.n.c. and of allatectomized females contained much glycogen, whereas the content of fat in the fat-body of females deprived of m.n.c. was generally smaller than in allatectomized females.

5. The accessory glands of females deprived of m.n.c. were of nearly the same length as those of allatectomized females.

6. The corpora allata of the females deprived of m.n.c. were definitely smaller than the corpora allata of the operated controls; this fact supports the assumption of an activation of the corpus allatum by the m.n.c.

7. Corpora allata of 7 days old females, when implanted into females deprived of m.n.c., had a slight effect on the growth of the ovaries, but were not able to bring about the development of ripe eggs.

8. M.n.c. from 7 days old females when grafted into females deprived of m.n.c. had a definite effect on the growth of the egg-chambers; this fact shows that the m.n.c. act on the ovaries by means of a hormone which they produce.

9. The lateral neurosecretory cells probably have a slight influence on egg development.

10. Flies are able to develop eggs in the absence of the corpus cardiacum; this shows that the neurosecretory cells and the corpus allatum alone are able to further the development of the ovaries.

11. The cutting of the two nervi oesophagi resulted in a retardation of ovarian development and in a hypertrophy of the fat-body.

12. The corpus cardiacum was found to have a similar effect on the growth of the ovaries as the m.n.c., but must be activated by the m.n.c. in order to exert its effect. The influence of the corpus cardiacum on egg development is due to a hormone released by this organ.

13. The stimulation of the corpus cardiacum-allatum system by the neurosecretory cells is partly hormonal.

14. *Thus in* Calliphora *the m.n.c. must be regarded as the over-all controlling centre of the endocrine system.*

15. It is suggested that the problem of the function of the m.n.c. and the corpus cardiacum in the adult *Calliphora* may be regarded as a problem of growth.

16. The growth of the egg-chambers and of the accessory glands in the adult fly was studied. (The growth of the corpus allatum has previously been investigated by the author).

17. The growth of the same organs in flies fed sugar and water, but no meat ('sugar flies'), was compared with the growth of the organs of flies fed sugar, water *and* meat. The slight growth of the organs of the 'sugar flies' was ascribed to the protein content of the larval fat-body, and it was suggested that the breaking down of this fat-body is regulated by the side lobes of the ring-gland.

18. As the egg-chambers, the corpus allatum and the accessory glands of the flies deprived of m.n.c. were of nearly the same size as the organs of the 'sugar flies', a working hypothesis is put forward that the m.n.c. and the corpus cardiacum exert a profound influence on the protein metabolism of the fly. There is some evidence that it is the synthesis of protein which is concerned.

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